

A cautionary guide to university rankings

Shifts in this year's college rankings from *US News & World Report* tell us a little about quality and a lot about the essentially arbitrary nature of the exercise.

There will no doubt be dancing in the streets of Pasadena this week, following the news that the California Institute of Technology has leapfrogged above the usual Ivy League contenders to top the college rankings presented each year by the news magazine *US News & World Report*.

Such a party is due not because of the significance of the event itself — which is slight — but because everyone at Caltech seems to need a break. According to *US News*, classes at the science and engineering school can often last until 2 a.m. One inmate describes Caltech life thus: “Grades, social life, sleep: pick two.” The magazine had to delve back 15 years for an example of interesting non-curricular activity at the school. The example in question was the 1984 Rose Bowl football game between UCLA and Illinois, at which Caltech undergraduates — lacking a football team of their own — reportedly hacked into the scoreboard to make it read “Caltech 38, MIT 9”.

On the surface, at least, Caltech officials are managing to keep their excitement under control. “I don’t think the rankings are terribly meaningful, but they are useful,” vice-provost David Goodstein told *US News*. “It’s like winning the Rose Bowl. It doesn’t change the school or its characteristics, but it’s a nice thing to have. I don’t doubt that we’ll use it in advertising to attract students and impress donors.”

Potential students are certainly paying attention. For high-school students spending the end of their summer vacation in what is now the traditional way — in the basement, playing computer games and surfing the web — all roads now lead to <http://www.usnews.com/>

usnews.edu/college/corank.htm. The site has been swamped beneath the weight of visitors.

When they get to the site, they will discover that *US News* has explained and qualified its findings with admirable forthrightness. It points out, for example, that Caltech has surpassed last year’s joint winners — Harvard, Princeton and Yale — chiefly on account of changes not in school performance, but in study methodology.

This year, instead of simply ranking the schools in order of success in each category — for example, amount spent per student — and adding up the rankings, *US News* weights the institutional scores to reflect the extent of each school’s achievements in each category. Caltech, for example, spends an astonishing \$192,000 per student, more than twice as much as any of its rivals. It has a student-to-faculty ratio of 3:1, against 8:1 at Harvard, for example. By giving weight to Caltech’s overwhelming resource advantage, the new technique has propelled the smaller school into pole position.

Until next year, that is, when fresh methodological alterations will no doubt give *US News* a new winner and a fresh blast of publicity. Changing the method is good: if it stayed the same, the rankings probably would too, and where are the headlines in that? In any case, *Nature* recommends that the youth of America should pause for a while before making that fateful selection. Next month, the *Princeton Review* will publish its own highly scientific “party school” rankings, which are at least based on a survey of real students. Last year, the State University of New York at Albany came top, whereas Caltech was nowhere to be seen. □

Scandal at the Exploratory

A heap of public money may be misdirected unless the developers of a new science centre see sense.

Infuriatingly for some, many members of the public don’t want to have their relaxation interrupted by tutorials on scientific principles. That’s why nobody expects the media to devote much space or air-time to lectures about genes, electromagnetic radiation or the immune system, all of which are relevant to contentious areas of technology. And all of us — especially if accompanied by children — know the sensation after an hour spent in a museum that the series of displays and explanations is falling into an increasingly enervated cortex.

All credit, then, to those who slip a little appreciation — or possibly even understanding — of science into potentially unreceptive individuals by hands-on involvement in displays. Two of the most notable centres are the Exploratorium in San Francisco, California, and the Exploratory in Bristol, England, which is probably the leading example in Europe. Visit either and you will see a throng of adults and children almost unwittingly exploring the laws of electricity, magnetism and optics, encountering the luminescent traces of quantum chemistry, or learning about human physiology. The level of explanation may not always be ideal, but the ‘plores’ triumphantly embody an approach that needs to be preserved. They communicate

the fundamentals of science, rather than its applications or implications, by engaging the visitor.

Scandalously, the Bristol Exploratory will close at the beginning of next month (see page 804). Having been used as the basis of a successful bid for £41 million (US\$65 million) from Britain’s national lottery to build a new science centre, to be called “Explore at Bristol”, and having set aside its own fund-raising efforts to support the new centre as, not least, its future home, the Exploratory finds itself out in the cold and without the funds required to continue. Those visiting a preview in Bristol of what the new centre will contain will see their worst fears confirmed: the organizers have settled for high-tech, screen-based demonstrations that make minimal effort to engage and stimulate the mind, and provide little explanation of scientifically shallow displays.

It is essential that scientific substance be incorporated into the new centre. Although it needs to have a broader appeal than a solely hands-on science centre can provide, Explore should incorporate the philosophy and exhibits of the Exploratory, so that the ideal of promoting understanding through fun can be sustained. Anything less would be to throw away a gem of scientific culture. □



Wellcome Trust loses battle to expand its genome campus

[LONDON] The UK government has rejected a proposed £100 million (US\$160 million) extension to the Wellcome Trust's Genome Campus in Hinxton, Cambridgeshire.

The proposal was seen as an important test of how the British government would balance its commitment to biotechnology 'clusters' with its promises to protect the rural environment.

The Department of Trade and Industry (DTI) has been emphasizing the importance of such clusters, as well as stronger links between universities and industry, since the launch of its policy paper on competitiveness at the end of last year (see *Nature* 396, 714; 1998).

The decision followed an extended — and bitter — battle between the Wellcome Trust and local planning authorities over the development of the site, which is home to the Sanger Centre for gene sequencing, the European Bioinformatics Institute and the Human Genome Mapping Project Resource Centre. The trust wants space to commercialize research from these laboratories. It says that locating biotech companies alongside researchers will help to generate ideas and make it easier for scientists who go into business to maintain links with their former laboratories.

But the proposals met with stiff opposition from the local authority and residents of nearby villages over the scale of the development, which was said to be a threat to the rural environment. The local authority argued that space for 'spin-out' companies already exists on the district's many science parks.

Despite promising a statement on the planning decision, the Wellcome Trust is refusing to comment until its governors meet on 8 September. But the news will come as a profound disappointment to the trust, which has repeatedly affirmed its belief in the proposal. In April, Mike Dexter, its director, complained about the length of time they had been kept waiting and said the government could aid biomedical research by ensuring that planning decisions were "taken in the national interest".

The trust must now consider whether to apply for a smaller extension, of just over half the 40,000 square metres originally requested, which would be likely to win planning permission but would not provide enough space for the expansion of biotechnology companies already on the site.

David Hussell, planning director of the local South Cambridgeshire District Council, said of the decision: "This is good news

for the planning system, which must strike the right balance between prosperity and environmental concerns."

The government also approved two smaller biotech developments in the Cambridge area, including an expansion of the Babraham Institute. Hussell contrasted the institute's approach with that of the trust as being "flexible" and hoped that the trust would "adopt a similar approach" in future negotiations.

The DTI rather optimistically described the planning decisions, which were taken by the Department of Environment, Transport and the Regions, as a "positive boost to the Cambridge biotechnology cluster". The rejection of the Hinxton Hall proposal, albeit

with the option for a smaller site, is seen as a snub for the DTI, whose science minister only this month published a report on promoting biotechnology clusters as part of a long-term strategy to promote their growth.

Successful clusters, said the report, "require concerted action across a range of policy areas, from supporting the science base to encouraging the flow of venture capital into companies and having urban planning policies that allow clusters to grow". The report notes that planning restrictions are a significant barrier to the growth of UK biotechnology and recommends that special planning zones be set up where clusters may develop.

Natasha Loder

Shoddy buildings cost lives in Turkish quake

[MUNICH] Seismologists and earthquake engineers say that the failure to implement building standards intended to cope with the risk of earthquakes contributed to the appalling death toll in last week's earthquake at Izmit in Turkey.

Earthquake engineers in Turkey and abroad say that many of the thousands of victims of the 17 August earthquake would have survived if new buildings had complied with local building standards.

The epicentre of the earthquake, which registered 7.5 on the Richter scale, was at the industrial port city of Izmit, 80 kilometres east of Istanbul. During the earthquake, parts of Turkey, where the Arabian and Eurasian tectonic plates meet, slid several metres to the west along the North Anatolian fault.

The risk of earthquakes along the fault, which stretches 1,300 kilometres from the Caucasus through northern Turkey to the Mediterranean sea, is extremely high. In 1939, an earthquake at Erzincan killed 45,000, and 1943, 1944, 1957 and 1967 all saw major earthquakes.

But the densely populated region was unprepared. Thousands died in new apartment buildings built on unsuitable ground and made of low-quality concrete without appropriate reinforcement.

Nicholas Ambraseys, an earthquake engineer at Imperial College in London, says that the transition from the traditional building materials of timber and brick to concrete has led to a serious lack of workmanship in Turkey. As a result, recently constructed apartment buildings collapsed, while older bridges and buildings, as well as most of the historical minarets, survived.



Old buildings, like this mosque in Golcuk, were still standing as new ones collapsed around them.

Seismologists say that better scientific knowledge would not have lessened the scale of the disaster. Turkish Earth scientists and engineers are highly regarded and have good contacts with geologists and disaster researchers in Germany, Japan and the United States.

"We have done the most that we can," says Mustafa Erdik, head of earthquake engineering at the Bosphorus University in Istanbul. But "the existing building codes are simply not being applied".

The Turkish Ministry of Public Works and Settlement tried to improve compliance two years ago, but the legislation was resisted by the construction industry, and has not been passed by parliament.

Seismologists and engineers from several countries have rushed to Turkey to provide scientific support in monitoring aftershocks and assessing the stability of buildings and water quality. They expect a series of strong aftershocks in the region over the next few weeks.

Quirin Schiermeier

UK centre 'drops science for sensation'

[PARIS] A new science centre being financed by Britain's national lottery as a replacement for Bristol's popular Exploratory is facing criticism that its exhibits lack any serious scientific content.

Critics argue that the new centre, which is called Explore at Bristol, is forsaking science in favour of high technology and spectacular visual impact. Among the most vocal critics are scientists who were linked with the Exploratory, one of Britain's first science centres, which is closing its doors and transferring its exhibits to Explore.

The Exploratory consisted of exhibits, known as 'plores', that illustrated scientific principles, for example those of electromagnetism. The new centre, on the other hand, will boast crowd-pleasers such as a walk-in womb and a virtual-reality exhibit in which visitors can ride on the back of sperm from ejaculation to fertilization.

Sir Michael Berry, a professor of physics at the University of Bristol and a science adviser to the Exploratory, was unimpressed when he visited a preview of the new centre last week with his 11-year-old daughter. "The building and setting are wonderful. But when you go inside, it's actually very disappointing," he says. "I found it to be an intellectually tatty version of the Exploratory."

The Exploratory, along with a wildlife centre now called Wildscreen, formed the basis of a successful bid for a £41 million (\$66 million) grant towards the project from the

Millennium Commission, which receives money from the national lottery. The bid was put together by a consortium known as Bristol 2000, and now calling itself at-Bristol. The Exploratory will close next week, with the new centre set to open in spring. In addition, the Exploratory's staff of 30 will need to find new positions.

The Exploratory was created in the early 1980s by Richard Gregory, an emeritus professor of neuropsychology at the University of Bristol who had previously been involved in building a similar hands-on centre in San Francisco called the Exploratorium. Gregory has been involved in the creation of the new centre and says the idea behind it was to create an updated version of the Exploratory.

But Gregory and several of Explore's other scientific advisers complain that their opinions have been sidelined by the directors of at-Bristol. As a result, one of them says, the new centre lacks the "intellectual rigour" that distinguished the Exploratory. "We were supposed to be part of Explore but we got kind of rejected," Gregory says. "We found ourselves destroyed after 20 years."

About 30 per cent of Exploratory's exhibits will be on display in the new centre, with the rest being used for outreach programs or sold to raise funds. Explore needs to raise another £4.5 million to stay on budget.

Gregory says he hopes that more of the Exploratory will be incorporated into the new centre, but at-Bristol's directors say this



is unlikely. Nicholas Hood, chairman of at-Bristol, admits: "We haven't listened to [the scientific advisers] as closely as we should have." He adds that his organization has been "boxed into a remorseless calendar" in its bid to open soon after the Millennium.

Describing Explore's approach, Hood says: "Some people who are not as scientifically driven will be likely to say, 'wow'. And it's the 'wow' factor that we are searching for."

Colin Blakemore, a professor of physiology at Oxford and a member of Explore's scientific advisory committee, agrees with Hood that it was the time factor that excluded the scientists from the planning process. He hopes that future discussions with at-Bristol will eventually improve the scientific quality of the project.

Heather McCabe

Heather McCabe

A million volunteers join the online search for extraterrestrial life

[WASHINGTON] Three months after it began, an innovative scheme to enlist public help in the search for extraterrestrial intelligence (SETI) already has more than a million volunteers taking part in 224 countries — making it the largest distributed computing project in history.

SETI@home is a downloadable screensaver programme that uses idle time on home and office computers to look for patterns in radio-telescope data. Its spectacular success now has its creators thinking about ways that scientists in other disciplines could harness lots of small

computers to process large amounts of data.

The programme was initiated by the University of California at Berkeley's SERENDIP SETI project, which every day records 35 gigabytes of data collected by the Arecibo Radio Telescope in Puerto Rico. The idea behind SETI is to search all this radio noise for non-random patterns that might possibly be deliberate signals from another civilization.

But analysing large volumes of data with the desired sensitivity and frequency resolution is a daunting task. A distributed system “was really the only way we could solve this computation problem”, says SERENDIP director Dan Wertheimer. “Together, the million participants have formed the largest supercomputer on the planet.” The combined force is capable of six teraflops, or six trillion floating operations, per second.

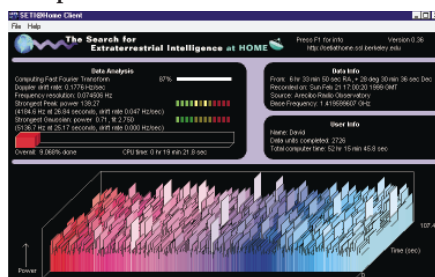
Participants download the screensaver programme from a Berkeley web server (<http://setiathome.ssl.berkeley.edu>) along with 350-kilobyte 'work units' representing 107 seconds of Arecibo data. When one unit

finishes processing, it is uploaded to Berkeley and another one is downloaded.

Wertheimer, who expected only 100,000 participants, admits that there were growing pains, including early difficulties handling the data traffic and providing customer support. He also says he was worried about quality control in the data returning from users. But very few people have been filing fraudulent work units, and these have been easy to spot. The project is expected to run for two years.

David Anderson, the Berkeley computer scientist who leads the project, says he is now looking around for other problems to work on that could benefit from such large-scale distributed computing. He has already had queries from biologists working in fields ranging from drug design to genetics, and hopes to set up a centre at Berkeley to offer the public a choice of projects in which to participate.

The challenge for scientists — and an obvious advantage for SETI — is coming up with computational problems that capture the public imagination. **Tony Reichhardt**



SETI goes home: a screensaver searches for patterns in intergalactic radio noise.

Leadership wrangle forces Salk Institute restructuring

[SAN DIEGO] The Salk Institute in La Jolla, California, is restructuring its administration amid staff divisions over the leadership of the world-renowned biology research centre.

The institute's board met on 4 August to consider recommendations by a management consultant who was brought in after Thomas Pollard was relieved of his role as chief executive officer in February. Pollard remains president of the institute, with board chairman Frederick Rentschler serving as interim chief executive.

This change and the subsequent interim arrangement have left the faculty deeply divided, insiders say. According to one of them, one-third of the staff support Pollard, one-third do not and the remainder are indifferent.

Some staff members fear that if Pollard leaves or is ousted as president, the Salk Institute may enter a period of leadership vacuum — a situation that has harmed the institute in the past.

"We may find it difficult to find anyone outside the institute willing to take on the job, given the past history," says Ian Trowbridge, a cell biologist and senior faculty member who supports Pollard.

Trowbridge says that what he terms Pollard's "demotion" was a "misstep" by the board.

Bartholomew Sefton, a molecular biologist and senior faculty member, says: "We are in disarray at the moment, regarding who is leading the institute.

"Authority is unclear. As a result, it is hard to make decisions that involve a lot of money."

Rentschler, the retired chief executive of a food corporation, seeks to minimize the impression of strife at the institute, saying he is unaware of any deep concern among the faculty members.

In a telephone interview from his Montana ranch, he praised the management consultant, Edward Hamilton of the Los Angeles firm Hamilton, Rabinovitz and Alschuler, for "a terrific report" that "grabs the essence of the Salk Institute".

Rentschler says that the board's executive committee will now begin to prioritize and implement Hamilton's recommendations. "The report has the potential to put the Salk Institute on a strong foundation in the next millennium," says Rentschler, who declined to release its contents.

All sides of the internal debate agreed about the report's potential, but some staff and a member of the executive committee have said that implementing change will be a



Trouble at the top: 'temperamental' senior staff have made the Salk Institute difficult to manage.

tough job, made more difficult by the dynamics of the institute's faculty.

The 50 active members of faculty at the institute include some of the world's finest biologists. However, the high self-esteem of some of these individuals has contributed to the current management difficulties, say insiders.

Since Pollard lost the chief executive role, no explanation has been offered to staff or the public. Asked why he lost the position, Pollard says: "It is too complicated to explain. It is best to wait until the governance issue is straightened out."

A cell biologist and member of the National Academy of Sciences, Pollard was chairman of cell biology at Johns Hopkins University in Baltimore, Maryland before taking over at the Salk three years ago. Since arriving, he has seen its annual philanthropic contributions rise to \$25.5 million, taking its endowment above \$100 million for the first time.

Despite his achievements, some senior faculty members remain unhappy. Interviews with a number of staff produced no major concerns about Pollard's performance, but a series of disputes that several faculty members describe as "petty". Some senior staff, for instance, are upset that most innovation grants were going to junior staff.

"It is unclear to anyone how to handle temperamental faculty," says Sefton, who has served as head of the faculty for part of his 25 years at the institute. "I'm not sure I have an idea how to either."

The dispute came to a head early this year, when a handful of senior faculty members called Rentschler threatening to quit if he didn't make an executive change, according to statements he made at a faculty luncheon. This prompted Rentschler to step in as interim chief executive.

Rex Dalton

Canadian report urges universities to make research earn its keep

[MONTREAL] Canadian universities and research councils are under pressure to commercialize research results or risk losing federal grants, following a critical report to prime minister Jean Chrétien.

The report, prepared by a subpanel of Chrétien's Advisory Council on Science and Technology, calls for a "bold new approach" to the commercialization of university research. It claims that existing practice is "resulting in lost investment opportunities, jobs and social benefits".

The report, *Public Investments in University Research: Reaping the Benefits*, proposes that all recipients of federal research funds should make a commitment to obtain the greatest possible benefit for Canada whenever their research produces a commercial gain — or risk losing further funds.

It suggests that universities should identify "innovation" as their fourth mission, in addition to teaching, research and community service. Innovation is defined as "the process of bringing new goods and services to market, or the result of that process".

The subpanel also proposes that funding bodies include innovation in their mission statements and use it as a criterion for awarding research grants.

Although science advisers have not always had much success at influencing policy in Canada, observers believe that parts of this report are likely to be implemented, as it reflects current government thinking.

The subpanel says that Canada lacks a coherent policy for safeguarding intellectual property at its universities. It suggests that the government should provide additional resources to help universities strengthen their commercialization capabilities. It also calls for a review of tax policy to encourage innovation, and an overall increase in federal funding for university research.

Some academic researchers were quick to attack what they see as the report's over-emphasis on commercial values.

"They're trying to foist on the universities something which belongs in a different culture," says John Polanyi, a 1986 Nobel Prizewinner in chemistry. "They really don't seem to be aware of the fact that they are asking for something which, if it were given to them, would do damage to the universities."

But David Strangway, a former university president who now heads the Canada Foundation for Innovation, says: "I don't think very many universities are actually going to change their mission statements, but you may see them getting more [commercially] aggressive."

David Spurgeon

California initiative to probe transgenics in large animals

[TAHOE CITY, CALIFORNIA] The University of California at Davis has created a centre for transgenic research in large animals, which it hopes will be the first of a nationwide system of university-based facilities advancing biomedical and agricultural science.

Opened last month with university funds, the centre will conduct research in pigs, sheep, goats and potentially cattle, all of which are being studied as sources of proteins, drugs and transplant organs. Its founders hope that the centre will maximize the dissemination of science in a field where many results are closely guarded by the corporations that pay for them.

Davis researchers say they intend to push for a network of four large-animal research centres based at universities across the United States. They see these centres — which would centralize expertise for other universities to use — as a way to save money in an extremely expensive field.

Most US universities use mice, which are cheap and easy to study, for transgenic research. As techniques and results have improved, researchers want to study larger animals. But a single experimental pig can cost more than \$100,000, a sheep or goat \$75,000, and a cow anything from \$500,000 to \$1 million.

"We want to provide the opportunity for researchers to do a variety of studies" from transgenics to cloning, says James Murray, a Davis geneticist who founded the centre with physiologist Gary Anderson. Both discussed the new centre last week at the university's second Transgenic Animal Research Conference at Tahoe City.

After three years of planning, the centre is being funded initially with about \$750,000 in campus funds, designated under a special programme for new initiatives. The centre is a collaboration between animal science, the veterinary school and the medical school. Already, researchers from the University of California campuses at San Francisco and San Diego are considering carrying out large-animal projects at the Davis centre. And the Davis team is recruiting researchers for new project grants.

Caird Rexroad, an associate deputy director of the US Department of Agriculture's Agricultural Research Service in Beltsville, Maryland, says he sees the concept as worthy of close study. Research in larger animals "needs to be more efficient to move it along," says Rexroad, a reproductive physiologist.

He adds: "There is a significant role for the public sector in this research to build knowledge to which a lot of people will have access."

R.D.



Troubled waters: the University of California at Davis has pledged to continue fundraising for its research centre by Lake Tahoe despite the plan for a rival facility on the Nevada shore.

University confronts new rival across Lake Tahoe

[TAHOE CITY, CALIFORNIA] Plans by the University of California to create a \$12 million research centre on Lake Tahoe are being complicated by the University of Nevada's decision to build a duplicate centre on the other side of the lake.

The Tahoe Research Group at the University of California at Davis has been studying the ecology of the Tahoe basin, in the mountains between California and Nevada, for 41 years. Limnologists from around the world have used the group's research to address issues of algal growth, lake clarity and pollution.

But earlier this month, the University of Nevada at Reno shocked Davis officials by announcing a plan for a \$10 million research centre across the lake. Nevada researchers did little work on Lake Tahoe until a couple of years ago, Reno scientists acknowledged, although they have studied the soil and dying forest around the lake.

Charles Goldman, an internationally respected limnologist who directs the Tahoe Research Group, says he was "surprised, to say the least" by Nevada's plans. He added that Nevada's efforts have caused "anxiety" among his philanthropic donors — who have pledged to contribute about half the money needed to rebuild Davis's labs, currently housed in a former fish hatchery built in 1916.

"It is making it more difficult to complete our fund-raising, and it's bad for the science," says Goldman. "But regardless of what Nevada does, we will raise the funds and continue our research."

Davis officials also say that scarce government funds for research on restoring Lake Tahoe will not be used efficiently if duplicate overhead costs are needed for the proposed Nevada facilities.

But Glenn Miller, who directs the University of Nevada's Center for Environmental Sciences and Engineering in Reno and is involved in planning the lake facilities, says his university wants to play a more active role in studying Lake Tahoe. "There is some value in diversity," says Miller, an environmental chemist. "We have different expertise."

Nevada began planning its centre more than two years ago, when President Bill Clinton and Vice-President Al Gore visited the lake and called for more ecological restoration efforts. As Davis and Nevada scientists subsequently discussed ways to improve lake ecology, Nevada officials were discreetly planning their own facility, garnering support from an unnamed Reno foundation which will donate the \$10 million.

The University of Nevada plans to build a research facility, conference centre and offices on federal land on the Nevada side of the lake near Round Hill. A smaller research facility is also planned for a second site nearby that is to become public land.

Miller — a Davis graduate — acknowledges some embarrassment at the rivalry between the two universities. When Nevada's plans were revealed this month at a political meeting between representatives of the two states to discuss Lake Tahoe restoration, some University of Nevada officials denigrated the Davis plans, telling the press that they didn't "have to be friendly" with the California team. Miller calls such comments "stupid," lauding "top-flight research" by Goldman and his team.

Some observers wonder why Nevada did not combine forces with Davis. But it seems probable that the University of Nevada wants to boost its share of government research funds associated with a proposed \$900 million restoration of the lake.

Rex Dalton

Astronomy adapts to sharpen up its image

Tony Reichhardt

By correcting for atmospheric distortion, adaptive optics – despite some birth pains – gives telescopes a far clearer view of the Universe.

[WASHINGTON] The Keck Observatory in Hawaii will gain an important new capability this week when guest astronomers start using its long-awaited, \$7.5 million adaptive optics (AO) system. A team led by Shri Kulkarni of the California Institute of Technology will be the first researchers to use the system.

Like all such systems, Keck's corrects for atmospheric turbulence by sensing how light is distorted by the shifting air above and countering the distortion with a deformable mirror. The result is an order-of-magnitude improvement in image sharpness, with resolutions down to the diffraction limit, the telescope's theoretical performance limit.

The Keck AO system saw its first light in February, and has already produced images with resolutions of 22 milliarcseconds at a wavelength of 850 nanometres, very close to the diffraction limit.

The debut of adaptive optics on the ten-metre Keck II, the world's largest single telescope, and its introduction at other observatories in coming months, mark the technology's coming of age. This follows years of promises and the recent commencement of productive science using four-metre-class telescopes. Adaptive optics is "close to being ready for prime-time extragalactic astronomy", says Chris Shelton, Keck's instrument scientist for AO.

Differing approaches

Other large instruments about to start using AO systems include the eight-metre Gemini North telescope in Hawaii — already getting impressive results with an interim system designed at the University of Hawaii — and the Multiple Mirror Telescope (MMT) in Arizona. The 6.5-metre MMT will use a secondary mirror that is itself deformable, increasing the system's sensitivity. Project managers hope to have the refurbished telescope and AO system running by next spring, according to Michael Lloyd-Hart of the University of Arizona's Steward Observatory.

In October, Steward astronomers plan to place a sensitive spectrograph behind the AO-equipped 3.5-metre telescope at the US Air Force's Starfire Optical Range in New Mexico. Unlike most adaptive optics systems built for astronomical use, which are optimized for infrared observation, Starfire's system operates at shorter visible wave-

lengths. This requires more correcting elements and is more technically challenging.

The air force has pioneered the use of adaptive optics to improve visual tracking of satellites from the ground. Lloyd-Hart says that "people are not aware of the advantages yet" of combining AO with spectroscopy. He adds that the new device is "exactly the tool you need" for detecting the spectral signatures of planets orbiting other stars.

So far, only a few observatories have produced consistent results with adaptive optics, such as views of binary stars, asteroids and ring arcs around Neptune (see *Nature* 400, 731; 1999). The 3.6-metre Canada-France-Hawaii Telescope (CFHT) is one of the successful ones. Its approach, pioneered by François Roddier of the University of Hawaii, uses a 'curvature sensor' to determine how natural starlight has been refracted by the atmosphere.

All AO systems require a bright source of light near the target to calibrate atmospheric distortion at a given moment. Although stars as faint as seventeenth magnitude have been used for AO correction, many objects of astronomical interest do not have such a bright object nearby, and are effectively off-limits.

The most promising solution to this problem is to bounce a laser beacon off a known layer of the atmosphere and back to the telescope. In principle, this opens the entire sky to AO-corrected viewing. Laser guide stars have been demonstrated at several locations, including Starfire and the Lick Observatory in California.

But operational laser AO systems have been "desperately difficult" to develop, admits astronomer Jerry Nelson of the Uni-

versity of California at Santa Cruz. Nelson is the designer of the Keck Telescope and director of a new Centre for Adaptive Optics, which last month received a \$20 million grant from the National Science Foundation.

Keck has struggled with its laser system, which Shelton says is "researchy and cranky". It will not begin routine observation until next year. The Gemini project expects to solicit proposals for its system next month.

Delivering the goods

The other main drawback of adaptive optics is that it corrects for only a tiny area of sky at a time. One proposed solution is 'atmospheric tomography', which would characterize the turbulence in different layers of the atmosphere all at once, then combine the results and use several deformable mirrors to apply the correction.

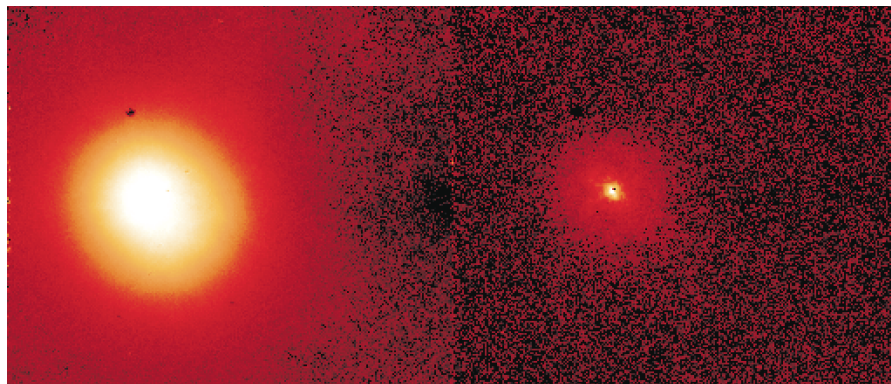
In theory, this could produce diffraction-limited observation for patches of sky as much as two arcminutes wide, compared with a few arcseconds for current AO systems. But the technique is even further in the future than reliable laser guide star systems.

The CFHT system can be operated by the observer, but more complex systems under development at Keck and elsewhere require at least two technicians. Most astronomers say the technology will not be considered truly operational until fewer people are needed to operate it.

But advocates of adaptive optics believe the systems are on the threshold of general acceptance. "It's been very, very challenging," says Nelson, but AO is about to emerge from being a "technological *tour de force* into a real scientific tool".

The new centre at Santa Cruz, involving 27 partner institutions, will carry that message to astronomers, familiarize them with the technology, and develop a general set of instruments and software tools for producing and analysing the resulting data.

Meanwhile, the cost of adaptive optics systems is coming down. One small Hawaii-based company, Laplacian Optics, reportedly has more than a dozen orders for complete, operational curvature-sensing systems of the kind being used on Gemini North. The price is just \$300,000 plus installation. □



First light: a star of magnitude 5.6 viewed through Keck, with (right) and without adaptive optics.

Shot in the arm for vaccine research as Gates gives \$6bn

[WASHINGTON] Bill Gates, the chairman of Microsoft, last week announced that he has given an additional \$6 billion to the Bill and Melinda Gates Foundation, the billionaire's charitable trust.

Some of the money is likely to be spent on research to develop vaccines for diseases including malaria, AIDS and tuberculosis. The foundation also aims to speed up access to existing vaccines, particularly in the developing world.

The \$6 billion donation brings the value of the philanthropy to \$17.1 billion, making it the largest in the United States. Earlier this year, the foundation gave \$75 million to vaccine research.

US Army funds institute of 'creative technologies'

[SAN DIEGO] The US Army has awarded \$45 million over five years to a new Institute of Creative Technologies at the University of Southern California (USC) in Los Angeles.

The institute will develop training programmes involving artificial intelligence, virtual reality and computer simulation. It

will involve researchers from several USC departments, and will work with entertainment companies to create training programmes that provide 'verisimilitude', a quality or state of appearing to be true, USC says. Within five years, USC hopes to have 200 researchers working at the institute, which will be located near Marina del Rey in Los Angeles.

Former CIA boss rapped for lapse in security

[WASHINGTON] John Deutch, the former head of the US Central Intelligence Agency who now teaches at the Massachusetts Institute of Technology, has been stripped of his CIA security clearance after admitting that he had kept classified files on an unsecured home computer.

The action apparently reflects the eagerness of the Clinton administration to appear even-handed in security matters. Groups representing Chinese-American scientists have complained that Wen Ho Lee, a former scientist at the Los Alamos nuclear weapons laboratory in New Mexico, was treated unfairly when he was sacked for careless handling of classified files.

Deutch's own careless handling of such files when he directed the CIA came to light after he was appointed earlier this year by

Bill Richardson, the energy secretary, to head a commission on the Los Alamos affair. His appointment was discreetly withdrawn when it emerged that classified files had been found on Deutch's home computer just after he left the agency in 1996.

Academy OKs EPA research on particulates

[WASHINGTON] More than a year after it recommended a large increase in US government research on the health effects of airborne particulate matter, a National Academy of Sciences panel has reported that the US Congress and Environmental Protection Agency (EPA) have risen to the task.

Following the panel's call for a 13-year, \$440 million research programme into particulate matter (see *Nature* 392, 642; 1998), Congress has increased funding, with a request for \$61 million next year, and the EPA has improved the scientific content of its particulate-matter monitoring programme. Despite the uncertainty surrounding a recent court ruling questioning the legality of the EPA's air particulate regulations, the panel holds the strong view that the research programme into particulate matter "should continue to move forward expeditiously".

Wiesel to head frontier science programme

[MUNICH] Torsten Wiesel, the former president of Rockefeller University in New York who won a Nobel prize in 1981 for his work on the physiology of vision, has been appointed secretary-general of the Human Frontier Science Programme. The programme supports intercontinental and interdisciplinary research in neuroscience and molecular biology.

Wiesel, 75, retired from Rockefeller last year. He will begin his three-year stint at the programme next April. One of his tasks will be to expand the programme's international funding base: at present, Japan pays 80 per cent of its costs (see *Nature* 399, 398; 1999).

Police probe 1953 death in nerve-gas tests

[LONDON] British police have launched a criminal investigation into the death of aircraftman Ronald Maddison following nerve-gas tests at the Porton Down chemical warfare centre in 1953.

The inquiry began after Wiltshire police received complaints from a former serviceman who participated in tests. Documents from the centre suggest that Maddison died in an experiment to

determine how much of the nerve gas Sarin was needed to penetrate a military uniform. It is also alleged that servicemen were tricked into taking part in the tests — being told the research was into a cure for the common cold.

The chairman of the House of Commons defence select committee warned last week that senior ministers were not properly informed about aspects of the current work at Porton Down.

Cave paintings off limits for maintenance work

[PARIS] Scientists and others will be excluded from the Lascaux caves in southern France, renowned for their prehistoric paintings, for at least a year while workers retune the systems monitoring the microclimate.

The Lascaux site, near Montignac, was closed to the public in 1963, but since then scientists or other important visitors have been permitted into the caves five days a week. Before its closure, the site received as many as 1,800 visitors a day. But the excess carbon dioxide created by so much traffic began to cause deterioration in the wall paintings, which date back 15,000 to 17,000 years.

The retuning of the monitoring system is expected to take more than a year. Only

two workers will be allowed in at a time to reset the systems that filter the air and maintain the temperature and humidity. Lascaux, which is the most well known of 130 prehistoric caves scattered across southwestern France, was discovered in 1940.

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Kansas makes a monkey of itself

Sir — When the journalist H. L. Mencken reported on the infamous Scopes trial of 1925, he remarked that the hysteria surrounding it had made a “universal joke” out of the occupants of Dayton, Tennessee, where the trial took place. Now, 74 years later, but only a few degrees of longitude removed, the Kansas Board of Education has in its turn made a monkey of itself. The board has removed the requirement for school students to have a knowledge of evolution to pass examinations (*Nature* **400**, 697 & 701; 1999).

This might seem hilarious in today's technically wired society were it not for one sobering fact. Despite overwhelming acceptance of the material benefits that science has brought, Americans in general remain deeply ignorant of its basic principles. If such ignorance persists, it will prove devastating to the future of our democracy whose citizens will increasingly

be called upon to exercise judgement on the complex social issues that advances in science inevitably bring.

As an illustration in the context of the Kansas decision, consider this — one of the most profound legacies of twentieth-century science for the next millennium is the discovery of the molecular structure of DNA in the 1950s. That provided the physical basis to begin to understand the processes of evolution on a genetic and cellular scale. In a few more decades, we will probably gain the knowledge to effect and accelerate, through directed genetic modification, the evolution of all life forms — including ourselves.

How we use this power, arguably the most potent ever to be possessed by humankind, will present the greatest social challenge to the survival of our species since the first primates stood up in the bushes, or, as they might now say in Kansas, since Adam awoke in Eden. We will have taken a big bite

out of the apple of the Tree of Knowledge, surpassing even that which released the secrets within the atomic nucleus. Whether we use the former to create the horror of a Huxleyan *Brave New World* or to enhance our basic humanity will be strictly up to us, just as is the decision on whether to use the latter for peaceful purposes or to bring about nuclear holocaust.

Thomas Jefferson, perhaps the prime champion among the founding fathers of the principle of separation of church and state, envisioned an American republic governed by a wise and educated electorate. To place at risk for the children of Kansas the chance to obtain all the vital knowledge that will enable them to keep Jefferson's dream alive in the coming age of biological revolution is both deplorable and terrifying.

Paul M. Grant

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Global emissions could soon start rising again

Sir — Some loose ends appear in your report of the Worldwatch Institute's estimates of global carbon emissions from fossil fuel use in 1998 (*Nature* **400**, 494; 1999). The industry report cited estimated that global emissions had fallen by 0.47% from 1997 levels, based on a US reduction of 0.2%. The US Environmental Protection Agency later estimated that 1998 US emissions had increased by 0.4%. This indicates that global emissions declined by 0.3%. The Worldwatch Institute claimed a 0.5% global reduction, despite acknowledging the +0.4% US figure.

In fact, the global reduction seems to have been more than 1%. This is owing to a reduction of more than 3% in the Asia-Pacific region and of more than 2% in the economies in transition in Eastern Europe and the former Soviet Union. Developing countries overall saw a decline exceeding 3% (though still up 33% on 1990 levels) because the decline in Asia more than offset small increases elsewhere. By contrast, emissions rose 1% in the European Union.

Reductions around the world were mainly the result of presumably temporary economic difficulties. In many cases they occurred in countries that are neither industrialized nor transitional economies listed in annex I of the climate convention and annex B of the Kyoto Protocol.

The Worldwatch Institute's belief that

the 1998 figures are “a sign that it may be less difficult to slow global warming under the Kyoto Protocol” than has been widely assumed, and the suggestion by others that voluntary reductions by industry will suffice, seem seriously complacent.

Michael Jefferson

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Alois Alzheimer and the amyloid debate

Sir — Peter St George-Hyslop and David Westaway discuss the possibility of using A β immunization to treat Alzheimer's disease¹. They mention the continuing controversy^{2,3} surrounding the role of extracellular A β accumulation and amyloid plaques in the causation of the dementia associated with the disease, referring to this as “an old debate”. What is not widely recognized is how old the question at the centre of this debate actually is.

Alois Alzheimer and his contemporaries⁴ considered this very question. In a 1911 paper entitled “On certain peculiar diseases of old age”⁵, Alzheimer writes: “There are cases of indubitable Dementia senilis, in which the plaques are not very numerous. Moreover, as Fischer stresses, they dislocate the nervous structures more than they destroy them. So the loss of cortical tissue due to the plaques cannot be very considerable. Furthermore, in places where plaques are not found in the cerebral cortex, we see the

well-known widespread senile sclerotic changes, the lipid-pigmented and granular degenerations of ganglion cells with alterations of their fibrils which Brodmann and Bielschowsky have described in detail, the fibre-formation of the glia, pigment-accumulation in the glia and the degenerative phenomena in the vessel-walls which it is impossible to believe were caused by plaques.

“These changes are found in the basal ganglia, the medulla, the cerebellum and the spinal cord, although there are no plaques at all in those sites or only isolated ones. So we have to conclude *that the plaques are not the cause of senile dementia but only an accompanying feature of senile involution of the central nervous system.*”

The italics are Alzheimer's and the translation is by Hans Förstl and Raymond Levy⁶. The paper was written by Alzheimer after his examination of only three patients with the disease. It is a remarkable testament to his meticulous work and originality of thought that 88 years ago he put forward an analysis that is not out of place in the context of the current debate.

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1. St George-Hyslop, P. & Westaway, D. *Nature* **400**, 116–117 (1999).

2. Hardy, J. H. *Trends Neurosci.* **20**, 154–159 (1997).

3. Davis, J. N. & Chisholm, J. C. *Trends Neurosci.* **20**, 558–559 (1997).

4. Bick, K. et al. (eds) *The Early Story of Alzheimer's Disease* (Liviana, New York, 1987).

5. Alzheimer, A. *Zschr ges Neurol. Psychiat.* **4**, 356–385 (1911).

6. Förstl, H. & Levy, R. *History of Psychiatry* **2**, 71–101 (1991).

Mechanics of the ribosome

Roger Garrett

Electron-density maps of a cell's protein-producing machinery – the ribosome – have been partially solved. This breakthrough shows that high-resolution structures of the whole ribosome will soon be available.

The ribosome is central to every cell because it provides the workshop and tools to synthesize all of the proteins (see Box 1). The simplest ribosome, from bacteria, is made up of two subunits known as 30S and 50S. These are defined by their apparent sedimentation coefficients, which are characterized by the Svedberg unit, S, and reflect the rate at which a molecule sediments in a solvent. Each subunit is made up of ribosomal RNA (rRNA), along with a large number of proteins. The 30S subunit, for example, contains a large rRNA molecule (16S rRNA) along with 21 proteins. The 50S subunit, by contrast, consists of large and small rRNAs (23S rRNA and 5S rRNA) and 35 different proteins.

On pages 833 and 841 of this issue, Clemons *et al.*¹ and Ban *et al.*² provide electron-density maps of the two ribosomal subunits, obtained by X-ray diffraction at resolutions of 5.5 Å and 5 Å, respectively. The 30S subunit comes from a hyperthermophilic bacterium, *Thermus thermophilus*¹, whereas the 50S subunit is from the extremely halophilic ('salt-loving') archaeon *Haloarcula marismortui*². For the first time, several proteins of known three-dimensional structure, and many regions of double-stranded rRNA, have been located in highly complex electron-density maps of ribosomal subunits.

This is not a sudden development. Crystals of these ribosomal subunits that diffract at high resolution have been available for years, and most of the crystallographic techniques used by Clemons *et al.* and Ban *et al.* have already been successfully tested on ribosomes³. Moreover, by using extensive biochemical data, most of the proteins and rRNA in each subunit have been incorporated into lower-resolution (in the 10–20-Å range) shapes of the ribosome determined by cryo-electron microscopy^{4–6}. For the 30S subunit, at least, this process has also exploited a spatial model of the ribosomal proteins that was derived from neutron-scattering data⁷. Previously, however, the X-ray data were not of a high enough quality to allow accurate models to be built.

The enormous effort that has gone into characterizing and analysing components of the ribosome^{5,6} has spawned many new experimental methods. It has led, for example, to a method for accurately predicting all

of the double-helical structures in rRNA⁸, including those incorporated in the new models^{1,2}. But progress in understanding how the ribosome works has been hampered by two developments — one avoidable and another that could not be prevented. Avoidable was the tradition of viewing the ribosome as two pieces of rock containing two (later three) shallow cavities where transfer RNAs (tRNAs; Fig. 1) carrying amino acids bind and interact. This produced the erroneous view that the tRNAs, the messenger RNA (mRNA) template and protein factors are located at one cavity or another, and that the ribosome itself is more or less irrelevant to the process of protein production. This view negated the concept of ribosomal movements. But we now know, from studies of cells containing mutated ribosomes with altered properties, and from investigations into how antibiotics block different steps of protein synthesis, that the ribosome is a dynamic machine^{5,6}. This evidence is strongly reinforced by physical measurements of the differences in shape observed for ribosomes in different functional states^{9,10}.

An unavoidable block to progress in solving the ribosome's structure was the fact

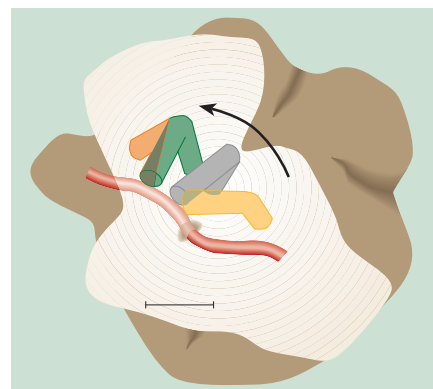


Figure 1 The ribosome, showing some of the states through which the tRNA–mRNA complex moves between the ribosomal subunits. AA, amino acid.

that, at a very early stage in evolution, the ribosome became highly complex (fossilized deposits of cyanobacteria and other bacteria and archaea containing ribosomes date back up to three billion years¹¹). Efficient proof-reading (to ensure that the mRNA template was followed correctly) and a fine balance between the speed and accuracy of peptide elongation developed. During this stage, the ribosome also defined the size of evolving genes, because the capacity to translate larger regions of mRNA more accurately allowed larger functional proteins to be produced. So, there is no simpler ribosome structure than the bacterial one (except for some highly degenerate ribosomes within the cell's respiration centre, the mitochondria). One consolation, however, has been that by comparing rRNA sequences, which are very similar ('conserved') between different organisms, reliable evolutionary trees of microorganisms

Box 1: Building proteins

Protein synthesis starts on the 30S subunit. The messenger RNA (mRNA) – the template for a new protein – binds to this subunit then unfolds its highly twisted structure as it enters. A transfer RNA (tRNA) molecule carrying the first amino acid for the new protein chain at one end (its so-called 3' terminus) binds to the 30S subunit and to the mRNA. It attaches to the latter by base-pairing to a sequence of three nucleotides in the mRNA template (a 'codon') that specifies which amino acid needs to be added.

Next, the 50S subunit associates with the 30S subunit, and the 3' terminus of the tRNA is positioned in a cavity on the large subunit. Another tRNA is bound at an adjacent codon carrying a second amino acid, which is also positioned in the cavity. A peptide bond is then formed to join the two amino acids. During each of these steps – which are repeated until a protein chain (polypeptide) is synthesized – the many protein factors that bind to the ribosomal subunits ensure that the

tRNA and mRNA molecules are positioned accurately, and they facilitate movement of the tRNA–mRNA complexes relative to the ribosome.

As can be seen from Fig. 1, the 3' end of each tRNA moves around 100 Å through the ribosome. When the new polypeptide is ready, another protein factor recognizes a specific 'stop' codon, allowing the protein to be released along a channel through the 50S subunit. The two ribosomal subunits then dissociate.

R.G.

could be constructed for the first time. This led to the discovery¹² of the third domain of life, the Archaea, in 1977.

What do the latest crystallographic results actually tell us about the ribosome? They put the whole model-building exercise on a surer footing, and promise much more. At this resolution, α -helices (spirals) in the protein structures can be readily fitted to the electron-density maps of the subunits, as can most double-helical segments (around two-thirds) of the rRNA's structure. Moreover, known three-dimensional structures of proteins and protein-rRNA fragments are placed with some confidence. Clemons *et al.*¹ have even pinpointed the position of a protein called S20 based on the α -helical composition that was predicted from its amino-acid sequence. In all other cases, though, to localize components of unknown structure (and this still includes two-thirds of the proteins in the 30S subunit and most of the proteins in the 50S subunit), it is still necessary to fall back on data from biochemical studies and cryo-electron microscopy^{4,6}.

The central domain of the 30S subunit contains about one-third of the 16S rRNA, and produces a flat projection — the 'platform' — that modulates the passage of the tRNA-mRNA complex through the ribosome. This whole region has now been modelled by Clemons *et al.*¹, revealing new details of structures that may influence movement of the tRNA-mRNA complex. In the 50S subunit, Ban *et al.*² have located two main regions that regulate at least one protein factor (called elongation-factor G), which helps the tRNA-mRNA complex to move through the ribosome after the peptide bond has been formed (see Box 1). One of these regions is a small stretch of 23S rRNA; the other is a protein-RNA complex. Both of these regions have previously been isolated from the ribosome and analysed by NMR spectroscopy and X-ray diffraction^{6,13}. Conformational transitions in these regions can be inhibited by antibiotics or toxins, leading to reduced cell growth or even cell death. For example, the 23S rRNA segment is modified by several toxins, including ricin and α -sarcin, which inactivate the ribosome. The function of the protein-rRNA complex, on the other hand, is blocked by the antibiotics thiostrepton and micrococin¹³.

When I first saw these X-ray structures I thought that this is how it must have felt, for those involved, to see the final stones being placed on the first pyramid. But on reflection, although this analogy may be appropriate for the amount of collective effort that has been expended, it would also repeat the conceptual mistake that has plagued the ribosome field (more pieces of rock). The ribosome, together with its accessories, is probably the most sophisticated machine ever made. All of its components are active and moving, and it is environmentally

friendly, producing only GDP and phosphate. The advances in ribosomal modelling presented by Clemons *et al.*¹ and Ban *et al.*² should first be savoured. They should then signal that this is the time for new thinking, for the development of new techniques and for young scientists to get involved. The static models produced by cryo-electron microscopy and X-ray crystallographic studies will be a passing phase — the next decades will be dedicated to studying the machine's movements. □

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Astronomy

Life beyond the pulsar death valley

Alex Wolszczan

Rapidly spinning, strongly magnetized neutron stars, which reveal themselves to astronomers as radio pulsars¹, have been known for more than 30 years. Yet despite a steady progress in our understanding of the physics of these fascinating objects, to paraphrase a quote from a 1976 conversation between Sandra Faber and Jonathan Arons², "we know how pulsars pulse, but we do not know how they shine". This disappointing state of affairs is heightened by the astonishing discovery of the slowest radio pulsar detected so far, reported on page 848 of this issue³. The 8.5-second rotation period of pulsar J2144-3933 is so long that, according to our current thinking about the ways pulsars shine, this one should not exist as an observable object.

The key to producing pulses is the rotation of the neutron stars, which have masses similar to the Sun's but diameters of only 20 km or less. Pulsars expend their rotational kinetic energy by emitting electromagnetic radiation and a wind of relativistic particles, leading inevitably to a gradual slow-down of pulsar rotation. Luckily, this slow-down is measurable and, together with the rotation period itself, gives a useful estimate of a pulsar's magnetic field strength and age. Only a small fraction of the total energy lost is radiated away in the form of beamed radio emission, which appears to originate well inside the pulsar magnetospheres, in the regions located just over the magnetic poles. Pulsars are best described as 'misaligned' rotating magnets in which the magnetic poles do not coincide with the poles of rotation, so that the spinning pulsar produces a series of evenly spaced pulses. The pulses themselves are beams of radio waves emitted along the magnetic axis of a predominantly dipolar, huge ($\sim 10^{12}$ Gauss) magnetic field.

The detection of an 8.5-second radio pulsar is likely to send theorists back to the draw-

1. Clemons, W. M. Jr *et al.* *Nature* **400**, 833-840 (1999).
2. Ban, N., Nissen, P., Capel, M. S., Moore, P. B. & Steitz, T. A. *Nature* **400**, 841-847 (1999).
3. Yonath, A. *et al.* *Acta Crystallogr. A* **54**, 945-955 (1998).
4. Müller, F. & Brimacombe, R. J. *Mol. Biol.* **271**, 545-565 (1997).
5. Hill, W. E. *et al.* (eds) *The Ribosome: Structure, Function and Evolution* (ASM, Washington DC, 1990).
6. Garrett, R. A. *et al.* (eds) *Ribosomes: Structure, Function, Antibiotics and Cellular Interactions* (ASM, Washington DC, in the press).
7. Capel, M. S. *et al.* *Science* **238**, 1403-1406 (1987).
8. Fox, G. E. & Woese, C. R. *Nature* **256**, 505-507 (1975).
9. Pettersson-Landen, L., Fredriksson, M. G., Öfverstedt, L. G., Skoglund, U. & Isaksson, L. A. *Exp. Cell Res.* **238**, 335-344 (1998).
10. Agrawal, R. K., Heagle, A. B., Penczek, P., Grassucci, R. A. & Frank, J. *Nature Struct. Biol.* **6**, 643-647 (1999).
11. Pflug, H. D. *Syst. Appl. Microbiol.* **7**, 184-189 (1986).
12. Woese, C. R. & Fox, G. E. *Proc. Natl Acad. Sci. USA* **74**, 5088-5090 (1977).
13. Porse, B. T. & Garrett, R. A. *Cell* **97**, 423-426 (1999).

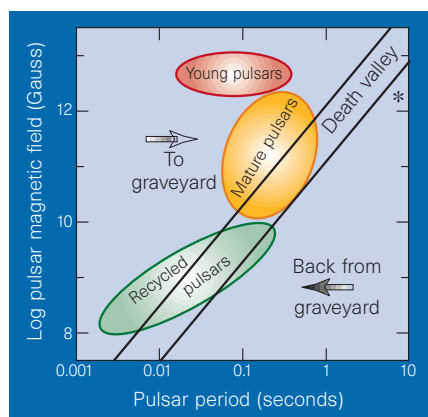


Figure 1 Life and death of a radio pulsar. The distribution of young and old pulsars is shown according to the strength of their magnetic fields and how fast they rotate. The two sloping lines define 'death valley', where pulsars are assumed to turn off their radio emission. Arrows indicate possible directions of pulsar evolution, with only superfast 'millisecond pulsars' coming back from the dead as recycled pulsars. The slowly rotating pulsar with a period of 8.5 seconds discovered by Young *et al.*³ (marked by an asterisk) does not fit in this picture.

ing board for another look at the details of the way pulsars shine. The most popular idea is that the observed radio emission arises from bunches of charged particles streaming out along the magnetic field lines. These particles are created over the 'polar caps' of the neutron stars and are subsequently accelerated to ultra-high relativistic speeds by a $\sim 10^{12}$ V electric-potential drop along the field lines close to the magnetic axis. If the magnitude of the potential drop is greater than a critical value, the pulsar will keep on pulsing away. In the lifetime of a pulsar, illustrated in Fig. 1 (see also Fig. 2 on page 848), a young pulsar will follow a well-worn path as it slows down (moving to the right in the graph). If the

magnetic field does not decay (it is unclear whether it does or not), the pulsar's evolutionary track will remain horizontal, otherwise its path will become curved in the direction of a decreasing field. As the pulsar period becomes longer, the mighty particle accelerator gradually loses its power. Ultimately, after some 10^7 – 10^8 years, when the maximum available potential drop becomes less than the critical value, radio emission from the pulsar will turn off — it will enter 'death valley'⁴ and disappear from the radio sky.

Evidently, pulsar J2144–3933 does not quite fit in the above picture. Although its rotation period is almost twice as long as the 5.1-second period of the previous record holder, the pulsar has managed to stay alive in the region of Fig. 1 reserved for the dead. Pulsar J2144–3933 also does not belong to the group of 'recycled pulsars'. These are the most rapidly rotating neutron stars — so-called millisecond pulsars — which succeed in coming back from the 'graveyard' in Fig. 1 by acquiring mass and angular momentum from a binary companion⁵. Pulsar J2144–3933, apart from its unusually long period, looks normal enough as far as its other properties are concerned. This includes the very narrow pulse profile and its polarization characteristics, both of which are in agreement with empirical models⁶.

The discoverers³ of this peculiar pulsar list several interesting ideas that may help unravel the mystery of its existence. The rotation period of the pulsar is similar to the periods of unusual X-ray pulsars equipped with superstrong ($\sim 10^{15}$ Gauss) magnetic fields^{7,8}. In principle, pulsar J2144–3933 could be related to these 'magnetars', despite the fact that the pulsar's own magnetic field is much weaker (2×10^{12} Gauss). Another possibility is that the geometry of the magnetic field lines in the emission region sufficiently departs from that of a simple dipole, meaning that the death valley can be moved towards longer rotation periods⁴. Finally, one can question our understanding of the basic properties of neutron stars, or postulate an alternative radio-emission mechanism. It is clear, however, that a really meaningful discussion of these and other alternatives will depend on new detections of very slow pulsars like J2144–3933.

The authors are probably right in predicting that pulsar J2144–3933 must be the tip of the iceberg of a sizeable population of slowly rotating pulsars that may be difficult to pin down. The newly discovered pulsar is intrinsically weak and has a very narrow emission beam, as expected of very old, slowly rotating neutron stars. For these reasons alone, it may be difficult to detect more such objects in the near future. Moreover, in the recent past, pulsar searches have been driven by a desire to find ever more millisecond pulsars that may offer glimpses of new and exotic phenomena⁵. In fact, the work by Young *et*

*al.*³ dramatically demonstrates how easy it is to overlook a boring 'vanilla flavour' object during a highly automated pulsar survey. As a result, the existing vast databases will have to be sifted through once again, this time with the sights set at the long-period end of the pulsar rotation spectrum. Similarly, new and continuing survey data will most certainly be analysed with the aid of algorithms that are sensitive to super-slow pulsars⁹. The detection of just one more object of this kind will certainly add a new, decidedly non-vanilla flavour, to studies of the pulsar emission mechanism. □

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1. Lyne, A. G. & Graham-Smith, F. *Pulsar Astronomy* (Cambridge Univ. Press, 1998).
2. Arons, J. *Space Sci. Rev.* **24**, 437–510 (1979).
3. Young, M. D., Manchester, R. N. & Johnston, S. *Nature* **400**, 848–849 (1999).
4. Chen, K. & Ruderman, M. A. *Astrophys. J.* **402**, 264–270 (1993).
5. Phinney, E. S. & Kulkarni, S. R. *Annu. Rev. Astron. Astrophys.* **32**, 591–639 (1994).
6. Rankin, J. M. *Astrophys. J.* **352**, 247–257 (1990).
7. Van Paradijs, J., Taam, R. E. & van den Heuvel, E. P. J. *Astron. Astrophys.* **299**, L41–L44 (1995).
8. Kouveliotou, C. *et al.* *Nature* **393**, 235–237 (1998).
9. Nice, D. J. *Astrophys. J.* **513**, 927–932 (1999).

Cognitive neuroscience

How do you pay attention?

Jeremy M. Wolfe

It has been dry in eastern Massachusetts and, as a consequence, we have been watering the tomatoes. There are two ways to water tomatoes. One is to take the hose or watering-can and go from plant to plant, watering each in 'series'. The other is to spray the entire patch, watering all the plants at the same time in 'parallel'. If visitors arrive after the job is done the tomato patch will obviously be wet, but it will not be clear whether the deployment of water was serial or parallel.

Although this serial/parallel distinction may be of minimal importance in the watering of suburban vegetables, it has been central in attempts to understand our ability to process visual information. Your eyes present your brain with more information than it can fully process. So, for example, even if the print was made large enough, you could not read this article at the same time as you read another. You must select, or 'attend', one

chunk of text. To understand how we see, we must understand how we attend¹. The results of the latest attempts to distinguish between serial and parallel modes of attention are reported by Woodman and Luck² on page 867 of this issue. These findings favour the serial view.

To appreciate the serial/parallel distinction in attention, look for the 'C' that opens to the left in Fig. 1. Although the letters are large and easy to see, you probably spent some time searching. You might have been deploying your attention from C to C in a serial manner. Alternatively, you may have been processing all the Cs in parallel — then your ability to identify the orientation of the C would have been slowed by the need to spread your resources over several Cs at once.

We can make various indirect measurements of this search process. For instance, on average, it takes longer to find a left-facing C among 20 other Cs than to find the same target among just ten Cs. Typically, it will cost about 50 ms to process each additional item³. Similarly, it would take longer to water 20 plants than ten plants, but this is true whether the watering technique is serial or parallel⁴. (That is, unless the hose has infinite capacity. In that case, all plants could be watered in an instant. No real hose has unlimited capacity, but some models of attention feature such processes.) Although there are some very sophisticated approaches to teasing serial or parallel conclusions out of indirect measures such as response time and accuracy^{5,6}, a firm answer has remained elusive. What we really want is a chance to see the attentional 'gardener' in action.

There are several ways to look at attentional effects in the brain. Using functional magnetic resonance imaging, for example, we can see a 'spotlight' of attention illuminating some parts of the field of view and not others^{7,8}. But the temporal resolution of the

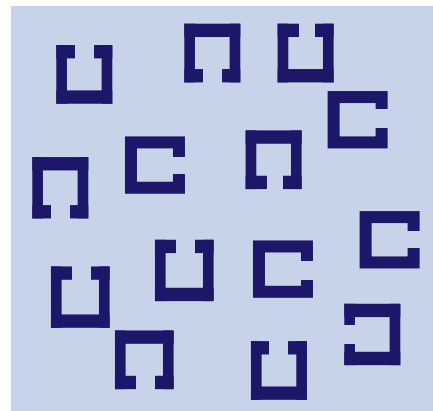


Figure 1 How do we see the C? When looking for a particular object — the left-facing letter C, for example — you may either process each letter in turn (attending in 'series'), or you might try to scan all of the letters at once (attending in 'parallel'). Studies by Woodman and Luck², using event-related potentials, indicate that we examine the letters in series.

method has been too coarse to distinguish serial from parallel deployment. Electro-physiological recordings from single neurons can also reveal the effects of attention^{9,10}, but they have not yet been used to assess the spatial and temporal dynamics of attentional shifts.

To try and visualize the deployment of attention, Woodman and Luck² used event-related potentials (ERPs) — scalp recordings of electrical activity in the human brain. ERP data are the changes in recorded voltage as a function of time since a certain event (hence the name 'event related'), and these waveforms have several useful attributes. First, their temporal resolution is fast enough to see shifts of attention that might occur within 50–100 ms. Second, their spatial resolution is at least adequate to tell the difference between stimuli in the left and right halves of visual space. Finally, an ERP waveform with the catchy name of N2pc has been shown to be associated with attentional selection¹¹. By examining changes in N2pc during visual-search tasks, Woodman and Luck believe they can prove that the mental tomato plants are being examined in series.

In one version of their experiment, subjects saw a field of rotated Cs. One of these Cs was red and another was green. Subjects were told that the target, if present, was three times more likely to be the red C than the green one. This should bias subjects either to attend first to the red C (if they are attending in series), or to allocate more attention to that item (if they are attending in parallel). Suppose that the likely (red) item is on the left side of the display and the unlikely (green) item is on the right, then consider what happens when the unlikely item is the actual target.

If attention is deployed in series, an N2pc response to the likely item should appear first in one cerebral hemisphere. It should be quickly followed by a response to the unlikely item in the other hemisphere. A model of parallel attention, on the other hand, might predict responses of different sizes to the different stimuli, but the time course of the response should be similar for both stimuli if they are both processed at the same time. In three versions of this experiment, Woodman and Luck found that the switch of the signal was consistent with serial deployment of attention. Moreover, the delay between the two N2pc signals agreed quite well with other estimates of the speed of serial attentional shifts.

Will one study resolve this long-standing controversy? Probably not. There are many parallel models of attention, and their supporters are very resourceful. For example, one could argue that attention is allocated to both items, but that more is given to the more likely item. The electrical signal of that attention would then become measurable at the likely location first. Although this one line of

hand-waving is not a theory, others are sure to appear before long offering more compelling 'parallel' accounts of this 'serial' finding. Nevertheless, even if Woodman and Luck's result turns out not to be definitive, it is a considerable advance because it places strong constraints on models of attentional allocation.

Why has it been so difficult to resolve the serial/parallel debate in the study of attention? Perhaps labelling attentional deployment as serial or parallel is like describing light as a particle or a wave — different experiments reveal particle- or wave-like properties of light. In the same manner, attention may appear serial or parallel to different experimental probes. As an analogy, think about a car wash¹². Cars enter and leave the car wash in series. However, at any given time, several cars can be washed. Is this a serial or a parallel process? One can imagine 'multi-lane' car washes that would further blur the distinction¹³. Seen in this light, Woodman and Luck's result illustrates one,

apparently irreducibly serial, aspect of the complex attentional machinery that selects some parts of the visual world for more extensive processing. □

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1. Pashler, H. E. (ed.) *Attention* (Psychol. Press, Hove, UK, 1998).
2. Woodman, G. F. & Luck, S. J. *Nature* **400**, 867–869 (1999).
3. Treisman, A. & Gelade, G. *Cogn. Psychol.* **12**, 97–136 (1980).
4. Townsend, J. T. *Psychol. Sci.* **1**, 46–54 (1990).
5. Palmer, J. *Curr. Dir. Psychol. Sci.* **4**, 118–123 (1995).
6. McElree, B. & Carrasco, M. *J. Exp. Psychol. Hum. Percept. Perform.* (in the press).
7. Brefczynski, J. A. & DeYoe, E. A. *Nature Neurosci.* **2**, 370–374 (1999).
8. Martinez, A. et al. *Nature Neurosci.* **2**, 364–369 (1999).
9. Gottlieb, J. P., Kusunoki, M. & Goldberg, M. E. *Nature* **391**, 481–484 (1998).
10. Treue, S. & Trujillo, J. C. M. *Nature* **399**, 575–579 (1999).
11. Luck, S. J. & Hillyard, S. A. *J. Exp. Psychol. Hum. Percept. Perform.* **20**, 1000–1014 (1994).
12. Wolfe, J. M. in *Fleeting Memories* (ed. Coltheart, V.) 71–94 (MIT Press, Cambridge, Massachusetts, 1999).
13. Harris, J. R., Shaw, M. L. & Bates, M. *Percept. Psychophys.* **26**, 69–84 (1979).

Biological oceanography

Complex lessons of iron uptake

Richard J. Geider

Oceanographers often speak of physical or chemical pressure on primary ocean productivity (that is, the photosynthetic conversion of CO₂ to new organic matter), but there is increasing evidence that marine phytoplankton also influence the fertility of the sea through production and consumption of dissolved organic compounds. This is the case for copper, which is detoxified by the release of specific organic ligands into the sea by planktonic bacteria¹. It is also true for iron dissolved in the sea, of which 99% is bound to organic ligands in a way that actually increases the availability of this essential nutrient².

Dissolved iron is present in very low concentrations in sea water (10⁻¹⁰–10⁻⁹ moles kg⁻¹) and in the absence of organic matter there would be even less, because free iron would precipitate at the surface as oxyhydroxides or be adsorbed onto sinking particles. So, organic ligands help to retain iron near the sea surface where there is sufficient light to support photosynthesis. But ligand-bound iron is a problem for species of phytoplankton that cannot transport these organic complexes across their plasma membrane, raising the question of how these organisms obtain iron. On page 858 of this issue, Hutchins and co-workers³ present convincing evidence that different groups of phytoplankton are capable of accessing iron that is tightly bound to organic matter using different mechanisms.

Iron was thought to be a potential limiting factor in marine productivity after studies of

plankton growth in the Southern Ocean early this century⁴. But it was only with the introduction of ultraclean sampling techniques in the 1980s that iron was shown to be a limiting nutrient, in at least 30% of the world's oceans^{5,6}. This was not always the case. Prior to the evolution of oxygen-producing photosynthesis about three billion years ago, iron was present at much higher concentrations in the ferrous (Fe(II)) oxidation state. With photosynthesis, the oxidizing conditions in the oceans led to ferric (Fe(III)) iron being precipitated, and a drop in the concentration of dissolved iron. In a perverse twist of fate this created an energy crisis for photosynthetic microbes, because large amounts of iron are used in both photosynthetic and respiratory electron-transfer chains⁷.

This energy crisis continues today in areas where iron is limited, particularly where light levels are low, such as the Southern Ocean⁷. Iron is also likely to be crucial to nitrogen-fixing organisms because the enzymes involved need large amounts of iron⁷. In the recent past, the drop in atmospheric transport of iron-laden dust from land to oceans during interglacial episodes may have been a contributing factor in glacial and interglacial variations in phytoplankton growth. Today, the oceans may be finely balanced between iron shortage and sufficiency, and increases in dust supply arising from global climate change could lead to sudden jumps in ocean productivity.

The nature of iron-binding ligands in sea water is poorly known, although there is evi-

dence for at least two functional types². Intracellular iron-binding molecules (such as porphyrin) are likely to be released into sea water following mechanical breakdown of cells. In addition, marine prokaryotes are known to actively excrete strong iron-binding compounds (such as siderophores), which are later reabsorbed with iron bound³. Although prokaryotes, such as cyanobacteria, produce siderophores, it is generally believed that eukaryotic phytoplankton do not. Previous work on laboratory cultures suggested that eukaryotes obtain iron exclusively from the dissolved inorganic iron pool (less than 1% of total dissolved iron in the sea)⁸. But this source of iron is unlikely to be enough to support the growth of eukaryotes. We need to understand how eukaryotic phytoplankton acquire iron because these larger organisms, in particular diatoms, play a dominant role in the carbon cycle in the oceans.

To shed light on this problem, Hutchins *et al.*³ compared the ability of cyanobacteria and diatoms to obtain iron from a range of organic ligands. The cyanobacteria tested are able to use iron bound to a variety of siderophores, but not iron bound to porphyrins. In contrast, the diatoms show a greater preference for iron from porphyrins than from siderophores. The authors suggest that uptake of bound iron by cyanobacteria may involve specific cell-surface siderophore receptors, whereas diatoms are thought to use a non-specific cell-surface enzyme (ferrioreductase) to liberate inorganic Fe(II) from the porphyrin complexes. Although this work clearly shows phytoplankton extracting iron from organic complexes, more studies are needed to characterize the putative ferrioreductase. This has practical implications because of the use of desferrioxamine (one of the siderophores examined by Hutchins *et al.*) to induce iron deficiency in high-iron ocean waters⁹.

Progress in understanding the influence of iron on phytoplankton growth⁵, the chemistry of iron in sea water², and the availability of organically bound iron to phytoplankton³ demonstrates that microbial activity modifies ocean chemistry, which in turn affects the ability of the ocean to support different microbial populations. This should come as no surprise, because other dissolved elements, including nitrogen and phosphorus, are often dominated by organic forms¹⁰. Although plausible biogeochemical models of nitrogen and phosphorus can be developed that do not include dissolved organic matter, this is not true for iron. Microbiology is the key to understanding the cycling of iron in the sea because microbes produce the organic ligands that keep iron in solution and possess the uptake mechanisms that transfer iron from dissolved to particulate phases. Moreover, the coupling of the iron cycle to the cycles of nitrogen, carbon and oxygen depends on the uptake of iron by catalysts for

nitrogen fixation, photosynthesis and respiration. This uptake can only be explained in terms of microbial physiology and population dynamics, which we barely understand.

There is still a great deal that we do not know about the responses of microbial ecology to climate change. The shortcomings in our mechanistic understanding of ocean microbiology are unfortunate because oceanographers are being asked to predict the interaction of global climate change with ocean biogeochemistry. If the processes that underpin biogeochemistry are controlled by factors that are only poorly understood, such as iron availability, then predictions based on past experience may be in error. □

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1. Moffett, J. W., Brand, L. E. & Zika, R. G. *Deep-Sea Res.* **37**, 27–36 (1990).
2. Rue, E. L. & Bruland, K. W. *Mar. Chem.* **50**, 117–138 (1995).
3. Hutchins, D. A., Witter, A. E., Butler, A. & Luther, G. W. *Nature* **400**, 858–861 (1999).
4. de Baar, H. J. W. *Prog. Oceanogr.* **33**, 347–386 (1994).
5. Coale, K. H. *et al.* *Nature* **383**, 495–501 (1996).
6. Behrenfeld, M. J. & Kolber, Z. S. *Science* **283**, 840–843 (1999).
7. Raven, J. A. *New Phytol.* **109**, 279–287 (1988).
8. Hudson, R. J. M. & Morel, F. M. M. *Limnol. Oceanogr.* **35**, 1002–1020 (1990).
9. Wells, M. L. *Limnol. Oceanogr.* **44**, 1002–1008 (1999).
10. Jackson, G. A. & Williams, P. M. *Deep-Sea Res.* **32**, 223–235 (1985).

Heart disease

Good cholesterol news

James Scott

Two decades ago, mutations in the gene that encodes the low-density lipoprotein (LDL) receptor were shown to cause familial hypercholesterolaemia — an inherited condition in which too much LDL ('bad' cholesterol) circulates in the blood. With this discovery came a new era in understanding cholesterol metabolism, leading to the development of powerful drugs to block the synthesis of cholesterol, lower the levels of LDL in the blood and help prevent coronary heart disease and strokes. By contrast, levels of 'good' cholesterol (the high-density lipoprotein, HDL) are inversely related to the risk of disease. Indeed, reduced levels of HDL are found in half of all patients with coronary heart disease. Despite this alarming statistic, our understanding of HDL metabolism has lagged behind that of LDL¹. But a crucial missing piece of the jigsaw is published in this month's *Nature Genetics*^{2–4} by three groups led by Michael Hayden, Gerd Schmitz and Gerd Assmann.

These groups have approached the problem by studying patients with Tangier disease. Named after Tangier Island in Chesapeake Bay — home to the isolated, settler community from which the first patients came — this is a rare, recessive disorder characterized by a reduced efflux of cholesterol from cells⁵. As a result, the macrophages (cells of the immune system that can ingest cell fragments) become engorged with cholesterol esters, and patients develop symptoms ranging from orange tonsils to coronary heart disease. Importantly, they have no HDL. The new studies show that Tangier disease is caused by defects in an ATP-binding-cassette (ABC) transporter gene, which encodes the cholesterol-efflux regulatory protein (CERP)^{2–4}. This discovery not only sheds light on HDL metabolism, but also provides opportunities for drug discovery and the prevention of heart attacks.

Genetic factors are important in determining the levels of circulating HDL. Premenopausal women, for example, have more HDL than men, which is why women have a lower risk of coronary heart disease. Levels of HDL can also be increased by exercise and weight loss, and even, albeit more modestly, by alcohol and by drugs such as fibric-acid derivatives, nicotinic acid and tamoxifen. The first stage of HDL biosynthesis is the assembly of nascent HDL from apolipoprotein-AI (apoAI), and phospholipid and free cholesterol discs, secreted from the liver and intestine (Fig. 1). In the mature HDL, the free cholesterol is esterified to form cholesteryl ester, which can then be either transferred to LDL or delivered to the liver and sterol-metabolizing tissues. In this way, cholesterol is removed from tissues, and cholesterol-engorged macrophages are discouraged from accumulating in the artery wall.

Normal cells pump out free cholesterol at a rate of 0.1% total cell cholesterol per minute. But patients with Tangier disease have a profound defect in this process, so mature HDL is not formed and apoAI is rapidly destroyed. The finding that the culprit is a defective ABC transporter (ABC1, also called CERP), is a milestone in understanding the generation of HDL^{2–4,6}. Hayden's group has gone even further and shows⁷ that a more common cause of coronary heart disease, familial HDL deficiency, is also caused by defects in CERP, and these results have been confirmed in *cerp* knockout mice³. So, Tangier disease joins the growing number of disorders — including cystic fibrosis, multidrug-resistant cancer and malaria, cholestasis (failure to secrete bile acids) and eye disease — caused by defects in ABC transporter genes. Intriguingly, patients with some of these disorders also have low levels of HDL.

The basic ABC transporter, found in both

prokaryotes and eukaryotes⁸, has four domains — two transmembrane domains and two cytoplasmic ATP-binding cassettes, which provide the energy for ligand transfer. The ABC transporters carry diverse ligands, including ions, amino acids, sugars, cytotoxic and anti-malarial drugs, phospholipids, sterols, vitamins, iron chelates and proteins. In the newly discovered CERP protein, the two halves of the molecule are linked by a long, charged, hydrophobic segment. A low-resolution structure of a typical ABC transporter, the multidrug-resistance P-glycoprotein, reveals a large aqueous chamber in the membrane, the walls of which are created by the transmembrane domains. This chamber has a large pore, which is open to the outside of the cell, as well as an opening into the lipid phase of the membrane (but not into the cytoplasm on the other side)⁹. Ligand might be transferred from the lipid phase to the aqueous chamber, then to an acceptor on the outside of the cell. Other, auxiliary proteins may be required to help transport bulky substrates.

The CERP transporter is probably made

in the cytoplasm of peripheral cells (Fig. 1) and then transferred to cholesterol rafts in the plasma membrane. Here, cholesterol increases the thickness of the lipid bilayers^{2-4,10}, favouring the segregation of proteins with long transmembrane domains to this location in the cell. The CERP probably transfers free cholesterol and phospholipid between the inner and outer leaflets of the plasma membrane. At the outer side, the cholesterol is then captured by apoAI for the production of HDL. However, we do not yet know the precise localization of CERP in the cell, or whether free cholesterol and phospholipid are its true ligands. The CERP transporter could transfer other ligands, such as a protein that facilitates the transfer of sterols and lipid.

Not all patients with Tangier disease have coronary heart disease, and Schmitz's group suggest³ that certain variants of the *CERP* gene may predispose to atherosclerosis by targeting cholesterol-packed macrophages to the artery. But based on the distribution of mutations in people with Tangier disease and familial HDL deficiency, there is no

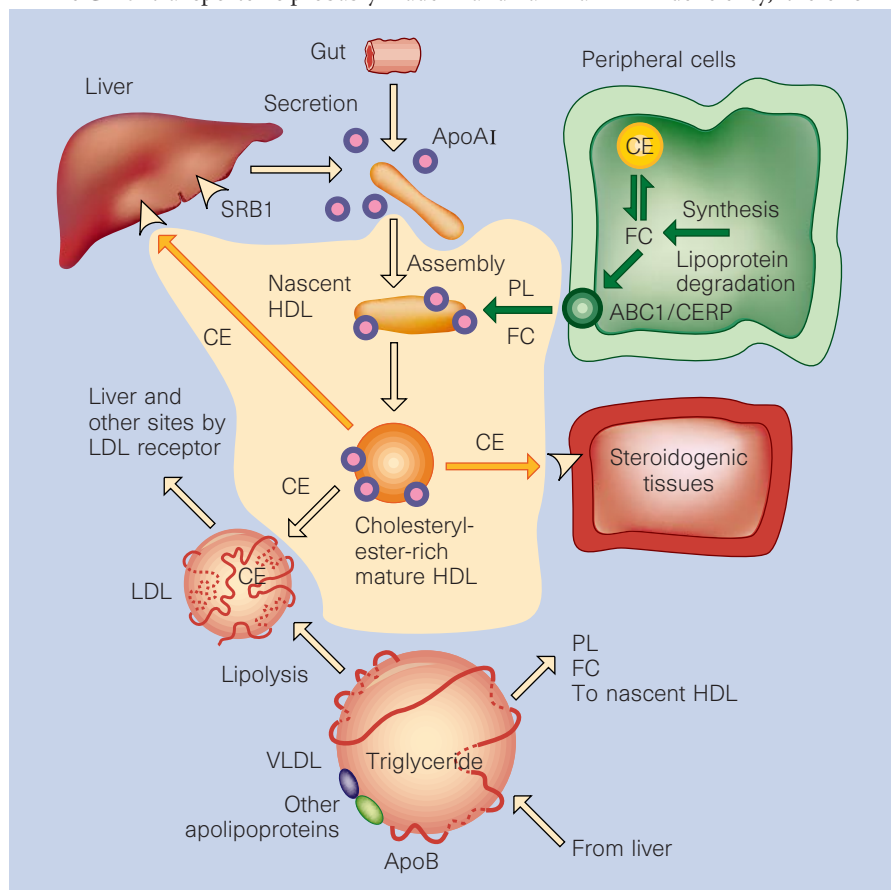


Figure 1 Cholesterol and Tangier disease. The newly discovered²⁻⁴ cholesterol-efflux regulatory protein (CERP) is necessary for the bulk transfer of free cholesterol (FC) and phospholipid (PL) out of cells. In the extracellular fluid, apolipoprotein-AI (apoAI) and nascent high-density lipoprotein (HDL) act as acceptors for the cholesterol. The free cholesterol in mature HDL is esterified (cholesteryl ester, CE) and transferred to low-density lipoprotein (LDL) and to cells by scavenger receptor B1 (SRB1). ApoB is the main apolipoprotein in LDL and very low-density lipoprotein (VLDL). So, in normal cells, ApoAI is recycled. In patients with Tangier disease, however, the absence of free cholesterol and phospholipid aborts the formation of HDL owing to defects in CERP. The apoAI is rapidly cleared from the circulation and degraded. Events that are defective in Tangier disease are shaded (light).



100 YEARS AGO

A few years ago a station was established near the town of San Marcos, Texas ... As the rainfall in western Texas is untrustworthy, the Commission determined to bore an artesian well to supply the water for its new station. ... Soon after the San Marcos well was opened a number of living animals began coming up with the water. So far, four kinds of Crustacea and a salamander have been seen, and of these quite a number have been obtained. The Crustacea are new to science and were described by Dr. James E. Benedict, of the Smithsonian Institution. They are white and perfectly blind. Most of the shrimps and crab-like animals have eyes set on the extremities of stalks that project above the surface. The shrimps from this well have the stalks, but the eyes have disappeared.

It is announced that Sir Edmund Antrobus is desirous of selling Stonehenge, the famous and mysterious monument on Salisbury Plain. Thinking it right that the nation should have the opportunity of purchasing this great relic of antiquity, the owner has offered it to the Government, with about 1300 acres of surrounding land (subject to certain pasturage and sporting rights), for the sum of 125,000/.

From *Nature* 24 August 1899.

50 YEARS AGO

Goethe, the greatest poet that Germany has produced, was a dominating intelligence who must claim a prominent place in any history of the human mind, and he devoted a considerable part of his effort to scientific studies. ... Yet we must face the fact that while there is in this scientific work of his much that is of the greatest interest, for the light that it throws on a superlative and complex character, there is not much — except, some would contend, in his botanical studies — that is important for the history of science. ... Goethe's scientific work may, perhaps, almost stand with Newton's work on theology and chronology — excellent, in many ways, if judged by the standard of the times, very important in the eyes of its producer, but not likely to have been remembered to-day had it been produced by a lesser man.

From *Nature* 27 August 1949.

structural support for this. The mis-sense mutations identified in *CERP* form a cluster around the ATP-binding cassettes at residues that are conserved in ABC1 orthologues from other species such as the mouse and the nematode worm. A similar clustering of mutations has been found in other genetic disorders of ABC transporters. As we identify different variants of *CERP*, we may find different effects on function.

And what about regulation of *CERP*? The intracellular concentration of cholesterol is tightly controlled owing to its importance in membrane metabolism, protein prenylation and the formation of steroid hormones. Expression of the genes that encode proteins involved in cholesterol biosynthesis and transport is regulated by proteins with sterol-responsive domains, which control gene transcription and protein turnover¹¹. Consistent with this, expression of *CERP* is upregulated by cholesterol. Yet levels of HDL are not decreased by the statins, which reduce intracellular levels of cholesterol. So, it is not clear whether *CERP* is regulated in a

similar way to other sterol-responsive genes. Nonetheless, its tight regulation and crucial role in generating HDL make *CERP* an excellent target for drugs. Small molecules that increase its activity — and, hence, levels of HDL — will almost certainly be found. The next step, therefore, will be to establish whether increased expression of *CERP* in transgenic mice protects against experimental atherosclerosis. □

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1. Fielding, C. J. & Fielding, P. E. *J. Lipid Res.* **36**, 211–229 (1995).
2. Brooks-Wilson, A. *et al. Nature Genet.* **22**, 336–345 (1999).
3. Bodzioch, M. *et al. Nature Genet.* **22**, 347–351 (1999).
4. Rust, S. *et al. Nature Genet.* **22**, 352–355 (1999).
5. Francis, G. A., Knopp, R. H. & Oram, J. F. *J. Clin. Invest.* **96**, 78–87 (1995).
6. Luciani, M. F. *et al. Genomics* **21**, 150–159 (1994).
7. Marcl, M. *et al. Lancet* (in the press).
8. Linton, K. J. & Higgins, C. F. *Mol. Microbiol.* **28**, 5–13 (1998).
9. Rosenberg, M. F. *et al. J. Biol. Chem.* **272**, 10685–10694 (1997).
10. Simons, K. & Ikonen, E. *Nature* **387**, 569–572 (1997).
11. Korn, B. S. *et al. J. Clin. Invest.* **102**, 2050–2060 (1998).

Cosmology

Shedding light on dark matter

Daniel E. Holz

Over 2,000 years ago, the Greeks thought they had it all worked out. In their cosmology, the entire Universe was composed of four elements: earth, wind, fire and water. Now, despite several millennia of effort, modern cosmologists are significantly worse off. We have no idea what the bulk of the Universe is composed of. We cannot even tell whether the majority of matter in the Universe is in some microscopic form (such as axions or other exotic particles) or in some macroscopic form (such as brown dwarfs or primordial black holes). Our ignorance of the mass of the basic building blocks of the Universe spans a good 50 orders of magnitude. In a paper in the *Astrophysical Journal* last month, Metcalf and Silk¹ propose that, by observing the gravitational effects of matter on the light from distant supernovae, it is possible to distinguish between microscopic and macroscopic forms of matter.

Although the idea of using supernovae as a cosmological probe of the matter distribution in the Universe has been suggested previously^{2–4}, Metcalf and Silk are able to include recent developments in our characterization of type Ia supernovae along with a full analysis of the gravitational effect of different distributions of matter on the observed supernovae brightness. In so doing they make a concrete proposal for a fundamental test of the 'dark matter' which, if successful, would be an important step forward in understanding the essential composition of the Universe.

There are strong reasons for believing that directly observable matter is a small fraction (less than one-tenth) of the total matter in the Universe⁵. The presence of dark matter, which does not reveal itself through the emission or absorption of photons, is inferred from its gravitational effects on other (directly visible) objects. As data in astrophysics and cosmology rely almost exclusively on the observation of photons (X-ray, optical, radio and so on), this leads to a catch-22: we predict the existence of dark

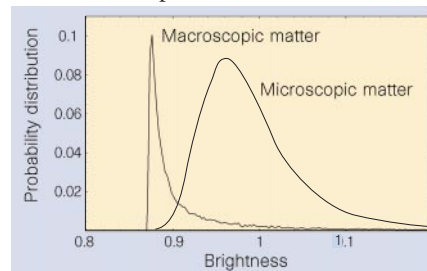


Figure 1 The observed brightness of supernovae at a redshift of one, corresponding to a time of explosion halfway back to the Big Bang. In a perfectly smooth, homogeneous and isotropic Universe, all such 'standard candles' would have a brightness of one. In a lumpy Universe, gravitational lensing by the intervening matter results in a probability distribution of observed brightness. Metcalf and Silk¹ have calculated two distributions: one for matter in macroscopic form (such as solar-mass black holes), and the other for microscopic matter (such as elementary particles).

matter because we don't see it directly, which in turn assures that the nature of this matter remains elusive.

Fortunately, gravity also interacts directly with light, in a phenomenon known as gravitational lensing. Imagine having many light sources of identical intrinsic brightness (these are called standard candles) placed at random directions in the sky, at a fixed distance from us. Now consider two extreme models for the distribution of matter in the Universe: either all matter is in microscopic form (such as elementary particles, perhaps clumped at the scale of galaxies, but no smaller), or it is entirely macroscopic (such as solar-mass black holes, clumped or not). Although the standard candles all emit the same amount of light, the observed brightnesses in each model will cover a range of values, owing to the clumpiness of matter in the Universe. In some instances the line-of-sight to a source might pass near or through a clump of matter (such as a black hole or galaxy), with the gravitational effects of the matter causing the photons to converge, resulting in a brightened image. In other cases the line-of-sight may pass far from any matter, so that the resulting image is dim. The precise distribution of the candles' observed brightness depends critically on the macroscopic or microscopic structure of the intervening matter (Fig. 1), and it is this skewed spread in brightness that Metcalf and Silk¹ propose to measure.

To detect this gravitational lensing one needs standard candles (if you don't know how bright an image is supposed to be, you can't tell whether it has changed in brightness). It is thought that we can tell the intrinsic peak luminosity of a type Ia supernova to within about 15%, making these objects excellent standard candles by astrophysical standards (Fig. 2). Metcalf and Silk ask how many such supernovae would need to be



Figure 2 A type Ia supernova (SN1994D) imaged with the Hubble Space Telescope. Supernovae brighten over the course of a couple of weeks, reaching a maximum brightness comparable to that of an entire galaxy, and then fade back to obscurity over several more weeks. The intrinsic peak brightness of a type Ia supernova is thought to be known fairly accurately, making them useful standard candles.

PETER CHALLIS, CENTER FOR ASTROPHYSICS

seen to be able to detect a spread in their observed luminosities as a result of gravitational lensing by dark matter. They find that with 50 supernovae at a redshift, z , of one (which corresponds to a time of explosion halfway back to the Big Bang), it would be possible to distinguish between microscopic and macroscopic dark matter with greater than 90% confidence. What makes this result particularly interesting is that two teams have been chasing supernovae^{6,7}, and between them have already observed several at $z \approx 1$. This data set has been responsible for one of the most exciting recent results in cosmology: that the expansion of the Universe appears to be accelerating⁸. As these groups continue their observations, they will certainly amass a large enough data set to make the Metcalf and Silk proposal viable.

Just as with the ‘accelerating Universe’ results, the greatest concern in the measurement of the gravitational-lensing effect is the robustness of the supernovae as standard candles. This property is at present a purely phenomenological observation. If it turns out that supernovae at high redshifts have a qualitatively different spread in peak brightness from ones nearby — although there is no indication of this so far — the Metcalf and Silk proposal would be infeasible. Even with 50 truly standard candles the lensing signal will probably remain inconclusive, as it is likely that the dark matter is in some combination of micro- and macroscopic matter, instead of the all-or-nothing models considered by Metcalf and Silk.

There are two possible ways to improve this method. By increasing the number of observed supernovae (to more than 100 at $z \approx 1$) it would be possible to determine the precise fraction of matter in different forms. Alternatively, by observing supernovae at greater distances one probes more of the Universe, thus obtaining a more pronounced lensing signal and allowing better discrimination between possible models. As they increase the number of supernovae detections, the observational teams are also pushing to higher redshifts (with a recent sighting at $z \approx 1.2$), and may be able to reach as high as $z \approx 1.6$. Looking to the future, Saul Perlmutter and collaborators have proposed a satellite, SNAPSAT, which would dramatically increase both the statistics (up to 2,000 supernovae per year) and redshift (up to $z \approx 2$) of observed supernovae. The lensing signal in such a comprehensive data set would provide a powerful probe of both the composition and structure of the dark-matter distribution.

Gravitational lensing is an ideal tool for studying the dark-matter composition of the Universe, as it uses the one property dark matter is assured to have: gravity. In the coming years supernovae may not only tell us how much of the matter in the Universe eludes our sight, but in what fundamental

form it is to be found. Perhaps we'll even be able to whittle our ignorance down to a mere one or two orders of magnitude. Two thousand years later we are poised to regain the level of certainty of the ancient Greeks. $\square$

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1. Metcalf, R. B. & Silk, J. *Astrophys. J.* **519**, L1–L4 (1999).
2. Linder, E., Schneider, P. & Wagoner, R. *Astrophys. J.* **324**, 786–793 (1988).
3. Rauch, K. *Astrophys. J.* **374**, 83–90 (1991).
4. Holz, D. E. & Wald, R. M. *Phys. Rev. D* **58**, 063501 (1998).
5. Turner, M. S. <http://xxx.lanl.gov/abs/astro-ph/9901109>
6. Riess, A. G. *et al. Astron. J.* **116**, 1009–1038 (1998).
7. Perlmutter, S. *et al. Astrophys. J.* **517**, 565–586 (1999).
8. Glanz, J. *Science* **282**, 2156–2157 (1998).

Signal transduction

Rhapsody in G proteins

Johannes L. Bos and Fried J. T. Zwartkruis

Guanine-nucleotide-binding (G) proteins are molecular switches that regulate a variety of cellular processes, transducing the signals received at a cell's surface to elicit a proper biological response from the proteins inside. They come in two flavours — the heterotrimeric G proteins and the small GTPases — and both can be regulated in two ways. The first involves the guanine-nucleotide-exchange factors (GEFs), which induce formation of the GTP-bound (active) form of the G protein. The second invokes the G proteins' intrinsic GTPase activity, which, by hydrolysing GTP to GDP, induces an inactive conformation. This hydrolysing activity can be greatly enhanced by GTPase-activating proteins (GAPs).

Mochizuki *et al.*¹ (on page 891 of this issue) and Jordan *et al.*² (in the *Journal of Biological Chemistry*) now describe an unexpected mechanism by which heterotrimeric G proteins can regulate a small GTPase called

Rap1. Heterotrimeric G proteins consist of α , β and γ subunits. In the inactive form, this trimeric complex is bound to a so-called seven-pass membrane receptor (or serpentine receptor) on the cell surface. When the receptor is stimulated, GDP is exchanged for GTP. The activated G protein then breaks free from the receptor, and the $G\alpha$ subunit dissociates from the $G\beta\gamma$ heterodimer. The new studies show that α -subunits of heterotrimeric G proteins directly associate with a GAP for Rap1, resulting in modulation of Rap1 and the extracellular-signal-regulated kinase (ERK).

Rap1 has recently attracted considerable attention. It is the closest relative of Ras — the godfather of the small GTPases — and is usually activated after stimulation of cell-surface receptors. These receptors may be linked to G_s proteins (which activate an enzyme called adenylyl cyclase) or G_q proteins (which activate phospholipase C)³, resulting in production of the intracellular

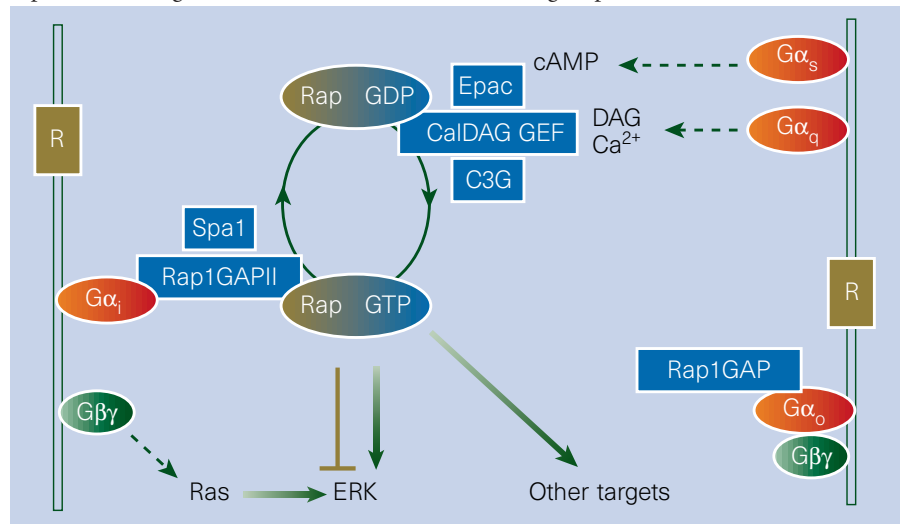


Figure 1 Positive and negative regulation of Rap1 by various heterotrimeric guanine-nucleotide-binding (G) proteins. Receptors (R) that couple to G_q through phospholipase C, and to G_s through adenylyl cyclase, generate the common second messengers cyclic AMP, diacylglycerol (DAG) and calcium. These second messengers bind to, and activate, Rap1-specific guanine-nucleotide exchange factors (GEFs). Mochizuki *et al.*¹ now show that receptors which couple to G_i inhibit Rap1 through direct binding of a Rap1 GTPase-activating protein (GAP) called Rap1GAPII to the α -subunit of G_i ($G\alpha_i$). Jordan *et al.*² show that the inactive form of $G\alpha_o$ binds to Rap1GAP, resulting in activation of Rap1. C3G and Spa1 are additional regulators of the Rap1 cycle. Active Rap1 may modulate activity of the extracellular-signal-regulated kinase (ERK), and additional targets are predicted.

second messengers cyclic AMP, calcium and diacylglycerol (Fig. 1). These second messengers then activate GEFs for Rap1 directly^{4,5}. For instance, cAMP directly regulates a protein called Epac, ('exchange protein activated by cAMP'), a result that is interesting in itself because it represents a protein-kinase-A-independent cAMP pathway⁵.

Mochizuki *et al.*¹ and Jordan *et al.*² now show that the α -subunits of two other G proteins — G_i and G_o , respectively — can form a complex with Rap1GAP. The authors detected this interaction by yeast two-hybrid analysis and by co-immunoprecipitation. There are, however, striking differences between the two papers. For example, Mochizuki *et al.* show that only an aminoterminal extended isoform of Rap1GAP, called Rap1GAPII, can interact with the active, GTP-bound form of G_{α_i} . The interaction leads to a decrease in the GTP-bound form of Rap1, indicating that the Rap1GAP is activated by the interaction with G_{α_i} . Jordan and colleagues, on the other hand, find that inactive G_{α_o} interacts with chicken Rap1GAP, resulting in the activation of Rap1. This implies that G_{α_i} and G_{α_o} use different molecular mechanisms to modulate GAP activity.

Mochizuki *et al.* further show that receptor-induced activation of G_{α_i} results in translocation of Rap1GAPII to the plasma membrane, and inactivation of Rap1. They also find that a mutant Rap1GAPII inhibits the receptor-induced inactivation of Rap1. Ever since GAPs were discovered, researchers have wondered whether, and how, they are regulated. Last year, Jiang *et al.*⁶ reported that stimulation of the $G_{\alpha_{12}}$ subunit via the thromboxane A_2 receptor leads to direct binding and activation of GAP1^m and inactivation of Ras. Mochizuki *et al.* now propose a similar mechanism for Rap1. Indeed, inactivation of GTPases by GAPs directly bound to the α -subunit of heterotrimeric G proteins may turn out to be a general phenomenon.

A frequently measured downstream effect of Rap1 is inactivation of ERK. This kinase is activated by many extracellular stimuli via the small GTPase Ras and the protein kinases Raf and MEK. Mochizuki *et al.*¹ show that when certain cells are stimulated with lysophosphatidic acid (LPA) — a growth factor in blood serum — Rap1 is inactivated, leading to activation of ERK and its downstream target, the transcription factor Elk-1. Both inactivation of Rap1 and activation of Elk-1 can be inhibited by a mutant Rap1GAPII, indicating that Rap1GAPII mediates the LPA-induced effects on ERK signalling.

This result was unexpected because LPA activates ERK through the $\beta\gamma$ -subunit of G_i , which, through a still-debated pathway, activates Ras⁷. The G_i -coupled receptors seem to activate ERK in two ways — by inducing a positive pathway ($\beta\gamma$ -mediated activation of

Ras), and by inhibiting a negative pathway (G_{α_i} -mediated inactivation of Rap1; Fig. 1). To add to the complexity, LPA (presumably through G_{α_q}) also activates Rap1. So, the overall effect on ERK may depend on the cellular and/or subcellular context. Indeed, activation of endogenous Rap1 does not always have an observable effect on the activity of ERK³.

Jordan *et al.*² used a different cell system in which Rap1 activates ERK. They show that introduction of G_{α_o} — but not an active mutant of this protein — can activate ERK. But it is not clear whether this activation is indeed mediated by Rap1GAP and Rap1.

Modulation of ERK activity may be just one function of Rap1, and other functions have been implicated. For instance, during development in the fruitfly *Drosophila melanogaster*, Rap1 is critical for regulating normal morphogenesis. This function, although ill-defined, is independent of Ras signalling⁸. Rap1 activity is, in fact, rapidly regulated by a variety of activated receptors, suggesting that it may act in a common process in receptor signalling³. One intriguing possibility arises from analysis of Bud1, the homologue of Rap1 in budding yeast. Bud1 is involved in recognizing a positional cue, and it recruits polarity factors (such as the Rho-like small GTPase CDC42) to position the actin cytoskeleton for the formation of a new bud. Bud2 — a GAP for Bud1 — may play a role in positioning Bud1, as well as in the essential cycling of Bud1 between the GTP- and GDP-bound states⁹.

So, maybe Mochizuki *et al.*¹ and Jordan *et al.*² witness the formation of a complex in which G_{α_i} or G_{α_o} is the positional cue to assemble a Rap1GAP–Rap1•GTP complex. This complex could perhaps, in turn, recruit polarity factors for the actin cytoskeleton. Such a function is not incompatible with the observed effects of Rap1 on cell morphology¹⁰ or with the inhibitory effect of SPA-1 (which is another Rap1-specific GAP) on receptor-induced cell adhesion¹¹. And it would not be the first time that yeast has set the stage for a molecular mechanism in mammalian cells. □

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1. Mochizuki, N. *et al.* *Nature* **400**, 891–894 (1999).
2. Jordan, J. D., Carey, K. D., Stork, P. J. S. & Iyengar, R. *J. Biol. Chem.* **274**, 21507–21510 (1999).
3. Zwartkruis, F. J. T. *et al.* *EMBO J.* **17**, 5905–5912 (1998).
4. Kawasaki, H. *et al.* *Proc. Natl Acad. Sci. USA* **95**, 13278–13283 (1998).
5. De Rooy, J. *et al.* *Nature* **396**, 474–477 (1998).
6. Jiang, Y. *et al.* *Nature* **395**, 808–813 (1998).
7. Luttrell, L. M. *et al.* *Curr. Opin. Cell Biol.* **11**, 177–183 (1999).
8. Asha, H. *et al.* *EMBO J.* **18**, 605–615 (1999).
9. Park, H. O., Bi, E., Pringle, J. R. & Herskowitz, I. *Proc. Natl Acad. Sci. USA* **94**, 4463–4468 (1997).
10. Kitayama, H. *et al.* *Cell* **56**, 77–84 (1989).
11. Tsukamoto, N., Hattori, M., Yang, H., Bos, J. L. & Minato, N. *J. Biol. Chem.* **274**, 18463–18469 (1999).

Daedalus

Sharp snow

Abrasives are an important but rather traditional technology. Grinding wheels, abrasive papers and loose powders all exploit tiny particles of grit made by crushing larger chunks of the material. These grains tend to be roughly spherical; few have any acute, sharp cutting edges. Worse, their orientation in use is entirely random. Only a tiny minority do anything to shape the workpiece at all.

Daedalus now wants to make sharp and spiky abrasive particles directly. He points out that gases conduct heat very badly. Thus a snowflake growing in air cannot readily get rid of its latent heat of condensation. Its points and edges are much better cooled, and therefore grow much faster, than its core. Hence the highly branched, dendritic form of snowflakes. Accordingly, to make an ideal sharp and spiky abrasive powder, condense it from vapour in a large mass of air or inert gas. Indeed, when some metals (such as zinc) burn in air, the resulting smoke consists of fine, highly branched and pointed single crystals of metal oxide.

DREADCO chemists are now exploring these ideas. Abrasives such as aluminium oxide might be made by burning the parent metal in carefully controlled conditions. But others, such as silicon and tungsten carbides, cannot. So the team is burning derivatives such as aluminium trimethyl, silicon tetramethyl, and so on, in limited concentrations of strongly preheated oxygen. The desired abrasive should form by reaction in the intensely hot flame. Its vapour will cool into a micro-snow of tiny, dendritic, sharp-edged, ferociously abrasive single crystals.

DREADCO's dendritic abrasives will transform the technology. No matter what its orientation, each particle will present one or more cutting edges to the workpiece; and lacking grain boundaries, the single crystals will be immensely strong. Grinding wheels formed from them will carve effortlessly through metal and masonry. Machinists, production engineers and even burglars will rush to exploit their irresistible cutting power. Dendritic abrasives will even make headway through modern engineering ceramics, many of which are so hard that at present they are almost useless. Taking his vision to its limit, Daedalus even dreams of striking a carbon arc in ultrahigh-pressure xenon. Formed under extremes of pressure and temperature, the resulting carbon vapour should condense into the most abrasive snow of all — dendritic diamond dust.

David Jones

Obituary

Eladio Viñuela (1937–99)

Pioneer of molecular biology in Spain

Eladio Viñuela, who died earlier this year in Madrid at the age of 62, was one of Spain's leading scientists. Beyond his own substantial research contributions, he was an inspiration to the Spanish scientific community, which has lost a key player in developing the country's strength in molecular biology.

Born in February 1937 in a small village in Extremadura, in southwestern Spain, Viñuela obtained his academic degrees at the University of Madrid before moving to the laboratory of Alberto Sols at the Spanish Research Council in Madrid. There he worked on carbohydrate metabolism, discovering the enzyme glucokinase, the synthesis of which depends on insulin and which is responsible for phosphorylation of glucose in the liver. He also demonstrated the allosteric properties of yeast phosphofructokinase, which has a regulatory binding site for ATP (the end-product of the pathway) that differs from the substrate site. The end-product inhibition of phosphofructokinase by ATP was proposed to act as a feedback control in the breakdown of glucose.

In 1964, Viñuela moved to New York University to work as a postdoctoral student in the laboratory of the Nobel prizewinner Severo Ochoa. At that time there was no molecular biology in Spain, so, to learn this relatively new area of biology, it was a must to move — preferably to the United States. This was a turning point in Viñuela's career. In New York he began collaborating with Charles Weissmann on research into the replication of bacteriophage MS2 RNA, especially the mechanism of parental-type 'plus strand' formation. This provided further insights into how MS2 RNA replicates. After this work he started to characterize the proteins induced after the infection of *Escherichia coli* with phage MS2, and to study translation of MS2 RNA. This was a time when the basic processes of the transfer of genetic information (replication, transcription and translation) started to be deciphered. The remarkable finding to emerge was that formyl methionine is the initiator of all the proteins encoded by the polycistronic MS2 RNA in *E. coli*, a result that quickly made its way into the textbooks.

But Viñuela's most influential work of this period was carried out independently,



This was the description that, in polyacrylamide gels in the presence of the detergent sodium dodecyl sulphate (SDS), there is a correlation between the electrophoretic mobility of a protein and its molecular mass. The technique was a revolutionary way of characterizing protein molecular mass, and much faster than the established method involving ultracentrifugation. The paper concerned was entitled "Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels" and appeared in *Biochemical and Biophysical Research Communications* (28, 815–820; 1967). It became a *Current Contents* citation classic, and the technique is still widely used to determine the molecular mass of proteins and to separate them according to size.

At the age of 26 Viñuela had married Margarita Salas, herself a highly talented molecular biologist. They had met during their university studies in Madrid and they both worked in the laboratories of Sols and Ochoa (the picture here shows them together in the lab). In 1967 they moved back to Spain to develop molecular biology and to contribute to science in their home country. They brought to Spain a new mentality and a new way of doing science, and together they created the first department of molecular biology of the Spanish Research Council in Madrid, starting with seven PhD students with backgrounds in biology, chemistry and medicine.

As researchers, Viñuela and Salas complemented each other well — her systematic thinking and his wide-ranging and restless intellect led them to achieve defined goals. Their investigations into the morphogenesis of bacteriophage ϕ 29 resulted in the identification of a protein,

covalently linked to the 5' ends of the viral DNA, that was later shown to act as a primer for ϕ 29 DNA replication. Protein priming turned out to be a hitherto unknown way to initiate replication in both DNA- and RNA-containing viruses.

From 1970, Viñuela used this basic research background to move into the study of African swine fever virus, an especially serious animal pathogen in southern Europe. Major contributions here were the description of the virus genome structure and replication strategies, as well as identification of the virus attachment protein involved in the interaction with the animal-cell receptor, and the discovery of multigene families in the virus genome. The complete sequencing of the viral DNA, and identification of several genes involved in the regulation of the host's immune response and apoptosis, provided telling insights into the biology of African swine fever virus. Basic studies of this virus are leading to more specific diagnostic tests for the disease.

Viñuela set an example through his own fruitful research and international reputation (he was among the first Spanish members of the European Molecular Biology Organization). He was also a catalyst in Spanish science in general, and in molecular biology and virology in particular, through his creation of an influential school of students. Many disciples of his are now senior research scientists and university professors in Spain and elsewhere; the passion for the scientific life and for rigour in research that they inherited from their maestro and *amigo* are transparent in *Phage ϕ 29 and the Origins of Molecular Biology in Spain*, a festschrift compiled to mark his 60th birthday. As a co-founder of the Centro de Biología Molecular 'Severo Ochoa' in 1975, together with Federico Mayor, D. Vazquez and A. Garcia-Bellido, it was his drive and vision that saw through the design and development of its technical department and core scientific facilities, a true innovation in Spanish research institutions at that time.

For current and future generations of Spanish researchers, Eladio Viñuela will remain a guiding light. His death on 9 March was untimely. Viñuela's erudition, capacity for organization and creativity together made him one of the best of scientists, and best of men.

Jesús Ávila and Federico Mayor Jr

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Journey into space

The techniques of perspective have achieved invisibility through omnipresence. Filippo Brunelleschi's Florentine peep-show captured the world in two dimensions and made reality virtual.

Martin Kemp

A historian of visual representation has an easy choice in choosing the act of the millennium — even though the two seminal works that testify to this act are lost. Probably before 1413, Filippo Brunelleschi painted two demonstration panels to show how to represent space and objects on a two-dimensional surface according to the systematic optical rules of perspective. In doing so, he established a mode of depiction that was to affect how images are conveyed in virtually every field of artistic, scientific and technological activity. By looking into the implicit 'boxes' of space behind the screens of our televisions or computers, we are distant legatees of Brunelleschi's vision.

The source for the lost images is an early biography of the Florentine sculptor, goldsmith, architect and inventor, particularly famous for his great dome of Florence Cathedral. The biographer, generally assumed to be his contemporary Antonio Manetti, describes how Brunelleschi charted the optical appearance of the octagonal Florentine Baptistery within its piazza from a vantage point within the central portal of the cathedral. He set up the resulting painting, which was about 30 centimetres square — "no miniaturist could have done better" — as a peep-show.

He drilled a conical hole in the panel at the point where the perpendicular line of his sight struck the Baptistery, and spectators looked through the reverse of the panel at a mirror held in front of the painting. The spectator could raise and lower the mirror to confirm the picture's accuracy.

The second panel represented another site of great moment to the Florentines, the Palazzo della Signoria, the seat of their government, as viewed diagonally from the corner of

The conceptual significance of Brunelleschi's invention was enormous. He established that there is a direct and verifiable relationship between something viewed from a particular point and its two-dimensional image on a plane surface — a canvas, panel, wall, sheet of paper and, most significantly, the page of an illustrated book.

the piazza. Since this panel was larger, Brunelleschi cut out the upper silhouette of the array of buildings so that the real sky could be seen above the painted roofs.

We do not know the projective procedures used by Brunelleschi — I am inclined to think that he adapted his mastery of practical surveying techniques — but Manetti tells us that Brunelleschi had invented what "came to be called *perspectiva*", the Latin term for the science of optics. The effect of Brunelleschi's invention on Florentine painting and relief sculpture of the following decade was revolutionary, not least in the fresco of the Trinity by Masaccio. A body of theory quickly gathered around the new science, including Leon Battista Alberti's seminal book, *On Painting*, and a treatise by Piero della Francesca, the painter-mathematician.

During the next two centuries, the painters' science was integrated into the sciences of projection by such mathematical luminaries as Federigo Commandino, Guidobaldo del Monte, Simon Stevin and Girard Desargues.

The conceptual significance of Brunelleschi's invention was enormous. He established that there is a direct and verifiable

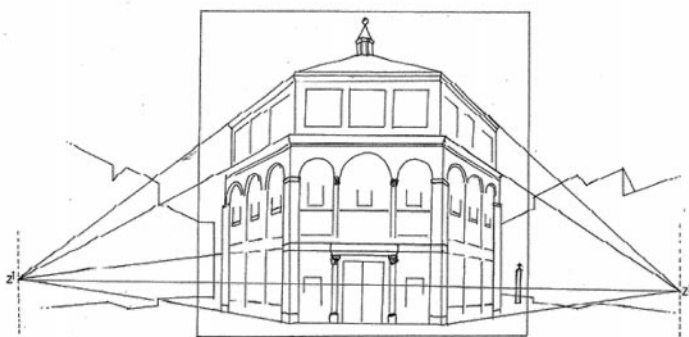
relationship between something viewed from a particular point and its two-dimensional image on a plane surface — a canvas, panel, wall, sheet of paper and, most significantly, the page of an illustrated book. A machine could be depicted to show how it would look to someone standing in front of it. Johannes Kepler's geometrical model of the cosmos could be shown as if it were a piece of divine clockwork. An animal could be portrayed standing solidly in a credible space. In short, anything could be represented in such a way that we seem to become surrogate eyewitnesses. The skilled image-maker persuades us to trust that what we are being shown has been portrayed, as they say, 'from life'.

But it was not all gain. Painters could use their technique to convince the observer that a depiction of something chimerical, such as a unicorn, recorded the real thing. An illusionistic picture hides aspects that cannot be seen from a single viewpoint, a particular disadvantage if we are seeking to understand a complicated piece of machinery. However, later variants of orthodox perspective, such as orthogonal and isometric — which retain consistent dimensionality rather than showing progressive diminution — allowed further dimensions of precise information to be conveyed.

Not least, as a tool for visualization, the perspectival system lets us imagine objects in measurable space. Artists and scientists gifted with high levels of spatial intuition can manipulate form and space by mental sculpting. And they can demonstrate their results through perspectival images.

Lost they may be, but Brunelleschi's demonstration panels rival any surviving artefacts from art and science for their effect upon what we see on a daily basis. □

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Reconstruction of Brunelleschi's perspective demonstration of the Florentine Baptistery (left) and the building today, pictured from a different angle.

Parasitoid behaviour and *Bt* plants

Transgenic crops that express genes targeted against insect pests may also affect non-target insects. For example, lacewings¹ and monarch butterflies² have been reported to be susceptible to toxins from *Bacillus thuringiensis* (*Bt*) that are expressed in *Bt* transgenic plants, although these results were obtained in small-scale laboratory assays in which insects were exposed to high levels of transgenically expressed toxin in no-choice tests. We show here that the behaviour of non-target insects can also play a part in determining how their populations will be affected by *Bt* plants.

Behaviour often influences the exposure of organisms to xenobiotics, and it is likely to be important for natural enemies of insect herbivores, whose primary route of exposure to engineered toxins will be through contact with hosts or prey feeding on plant tissues³. We have investigated this for a model system involving *Bt* oilseed rape (cv. Oscar, line O52) expressing the Cry1Ac toxin (which is highly active against many lepidopteran pests⁴), the diamondback moth (*Plutella xylostella*) and the parasitic wasp *Cotesia plutellae*. The wasp is a solitary endoparasitoid and an important enemy of *P. xylostella*⁵, itself notorious as the first insect pest to evolve resistance to microbial *Bt* sprays in the field⁶.

We find that, although parasitoid larvae forced to develop in *Bt*-treated susceptible moth larvae inevitably died with their hosts, behavioural factors are likely to limit the scale of this effect under field conditions. Furthermore, wasp parasitoids that had attacked *Bt*-resistant moth larvae on transgenic plants suffered no measurable adverse effects of *Bt* toxins on their behaviour as adults or on the survival of their larvae.

We investigated the performance of parasitoids against *Bt*-susceptible *P. xylostella* larvae by presenting them with young host larvae fed on transgenic (*Bt*) or wild-type oilseed rape for 4 h in 9-cm Petri dishes. In this no-choice situation, parasitoids oviposited into *Bt*-treated hosts, but the parasitoid larvae failed to complete their development. This was hardly surprising, given that all host larvae, whether exposed to parasitoids or not, died within five days of feeding on *Bt* plants — shorter than the minimum of seven days required by *C. plutellae* after oviposition to emerge from their host. In contrast, a new generation of adult parasitoids developed from 63% of *Bt*-susceptible moth larvae feeding on wild-type oilseed rape.

When parasitoids oviposited in larvae of a highly *Bt*-resistant laboratory strain of diamondback moth (NO-QA, originally selected with microbial *Bt*⁶) under the same conditions, there was no significant

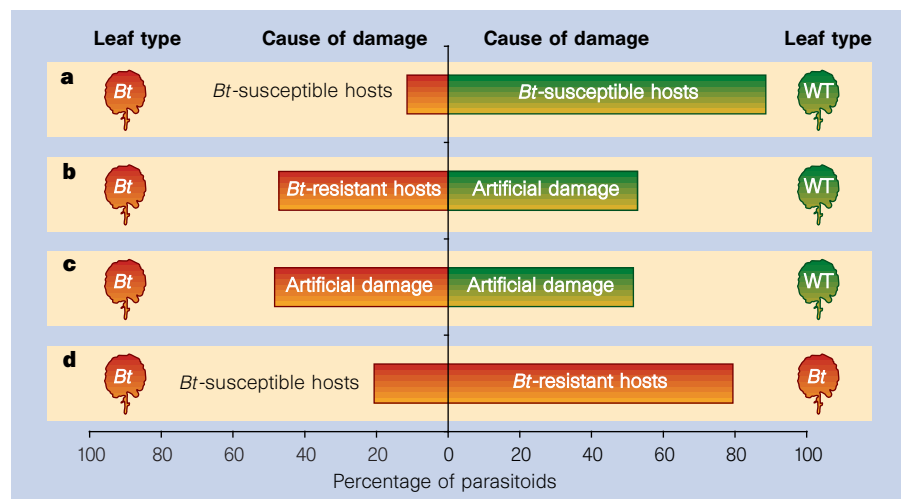


Figure 1 The percentage of parasitoids landing on *Bt* (brown) and wild-type (WT, green) oilseed rape leaves in choice tests in a wind tunnel. Parasitoids were released individually 70 cm downwind of two leaves placed 15 cm apart. Bars represent the percentage of parasitoids landing on each type of leaf. **a**, **b**, Host-damaged leaves were obtained by allowing two *Bt*-susceptible (**a**) or two *Bt*-resistant (**b**) diamondback moth larvae to feed on each leaf for at least 16 h. **c**, Leaves were artificially damaged by punching five 2-mm holes in each leaf. **d**, Attraction of parasitoids to *Bt* leaves damaged by either two *Bt*-resistant larvae or two *Bt*-susceptible larvae. About 40 parasitoid females were used for each bioassay.

difference in parasitoid survival in resistant hosts fed *Bt* or wild-type oilseed rape (54% and 56% parasitism, respectively; $P=0.868$). The presence of *Bt* toxin in resistant host larvae therefore had no direct effect on parasitoid survival, confirming previous results with microbial *Bt* formulations⁷.

Female parasitoids use herbivore-induced volatiles released from plants to locate their insect hosts, a behaviour that is critical for successful parasitism^{8,9}. We used a wind-tunnel technique¹⁰ to compare the flight response of the *C. plutellae* wasp towards *Bt* and wild-type leaves. For host-damaged leaves, *Bt*-susceptible or *Bt*-resistant *P. xylostella* larvae were allowed to feed on each type of leaf overnight. Flights were recorded as a choice if the wasp landed on a leaf within five minutes of take-off. The feeding damage inflicted by the hosts was measured after each bioassay.

The low consumption rate of *Bt* leaves by *Bt*-susceptible host larvae resulted in significantly ($P=0.005$) less feeding damage to the *Bt* leaves (mean, 9 mm²) than to wild-type leaves (mean, 138 mm²). When these leaves were placed in the wind tunnel, 89% of the parasitoids landed on the wild-type leaf and only 11% on the *Bt* leaf ($P<0.00001$) (Fig. 1a). No significant difference was found between feeding damage by resistant larvae on *Bt* and wild-type leaves ($P=0.33$), and the parasitoids did not distinguish between these two treatments ($P=0.43$) (Fig. 1b). Parasitoids did not prefer one plant type over the other if leaves were artificially damaged to the same degree ($P=0.50$) (Fig. 1c).

A further choice test compared *Bt* leaves damaged by either *Bt*-resistant hosts (feeding-damage mean, 101 mm²) or *Bt*-susceptible hosts (2.6 mm²; $P=0.0011$). In this test, 79% of the parasitoids flew to the *Bt* leaves damaged by resistant hosts, with only 21% choosing *Bt* leaves damaged by susceptible hosts, a difference that was highly significant ($P=0.00041$) (Fig. 1d).

Tactics aimed at suppressing pest populations risk disrupting the dynamics of their natural enemies by reducing host or prey density. These effects will be most pronounced for species (such as hymenopteran parasitoids) that tend towards greater ecological specialism and may even be specific to particular pest organisms.

Using *Bt*-resistant pests to evaluate direct effects of *Bt* toxins on parasitoid biology is a new way to assess ecotoxicological risk. The apparent lack of effect on the survival or host-seeking ability of the pest's enemy indicates that *Bt* plants may have an environmental advantage over broad-spectrum insecticides. The continued ability of *C. plutellae* to locate and parasitize *Bt*-resistant hosts on transgenic crops might even help to constrain the spread of genes for *Bt* resistance. Our results highlight the need to consider behavioural as well as toxicological aspects when looking at possible side-effects of transgenic crops on non-target organisms.

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- Hilbeck, A., Baumgartner, M., Fried, P. M. & Bigler, F. *Environ. Entomol.* **27**, 480–487 (1998).
- Losey, J. E., Rayor, L. S. & Carter, M. E. *Nature* **399**, 214 (1999).
- Schuler, T. H., Poppy, G. M., Kerry, B. R. & Denholm, I. *Trends Biotechnol.* **17**, 210–216 (1999).
- Stewart, C. N. Jr *et al.* *Plant Physiol.* **112**, 115–120 (1996).
- Talekar, N. S. & Shelton, A. M. *Annu. Rev. Entomol.* **38**, 275–301 (1993).
- Tabashnik, B. E. *et al.* *Proc. Natl Acad. Sci. USA* **94**, 12780–12785 (1997).
- Chilcutt, C. F. & Tabashnik, B. E. *Environ. Entomol.* **26**, 38–45 (1997).
- Turlings, T. C. J., Tumlinson, J. H. & Lewis, W. J. *Science* **250**, 1251–1253 (1990).
- De Moraes, C. M., Lewis, W. J., Paré, P. W., Alborn, H. T. & Tumlinson, J. H. *Nature* **393**, 570–573 (1998).
- Du, Y. *et al.* *J. Chem. Ecol.* **22**, 1591–1606 (1996).

Prelude or requiem for the 'Mozart effect'?

Rauscher *et al.* reported that listening to ten minutes of Mozart's music increased the abstract reasoning ability of college students, as measured by IQ scores, by 8 or 9 points compared with listening to relaxation instructions or silence, respectively¹. This startling finding became known as the 'Mozart effect', and has since been explored by several research groups. Here I use a meta-analysis to demonstrate that any cognitive enhancement is small and does not reflect any change in IQ or reasoning ability in general, but instead derives entirely from performance on one specific type of cognitive task and has a simple neuropsychological explanation.

Results from 16 studies on the effect of Mozart's music on the performance of cognitive tasks are summarized in Table 1. Meta-analysis² combining the effect sizes reported for all 20 published Mozart-to-silence comparisons (Table 1, top), involving a total of 714 subjects, yields an average cognitive enhancement of $d=0.09$ standard deviations, or only 1.4 IQ points.

Most of the tasks listed can be classified as stressing either 'abstract reasoning' (Raven's Advanced Progressive Matrices, Stanford-Binet matrices, backwards digit span) or 'spatial-temporal processing'³ (Paper Folding and Cutting, Revised Minnesota Paper Form Board). The Mozart effect for abstract reasoning is $d=-0.04$, whereas for spatial-temporal processing it is $d=0.14$ (or 2.1 IQ points). Accordingly, exposure to ten minutes of Mozart's music does not seem to enhance general intelligence or reasoning, although it may exert a small improving effect on the ability to transform visual images.

However, this enhancement is essentially restricted to a single task, is one-quarter as large as that originally reported for a broader class of cognitive abilities, is not statistically significant (combined $Z=1.14$, $P=0.26$), and is smaller than the average variation of a

single person's IQ-test performance (assuming a test reliability of 0.95, the 50% confidence interval would be 4.5 IQ points wide).

To account for the Mozart effect, Rauscher's group appealed to a model of cortical computation whose operations at the columnar level are compatible with qualities presumed to be present in Mozart's music and with the cognitive processing presumed to be involved in spatial-temporal tasks^{1,3,4}. But any improvement in performance with music can also be explained by the fact that the complex visual transformation processes involved in three-dimensional mental rotation and similar difficult spatial tasks (such as paper folding and cutting) are associated with function of the right cerebral hemisphere⁵, as is cognitive arousal^{6,7}.

In support of this, one study found that listening either to Mozart or to a passage from a Stephen King story enhanced subjects' performance in paper folding and cutting, but only for those who enjoyed what they heard⁸. Another experiment found that

8,120 British schoolchildren performed better in response to (presumably enjoyable) popular music than to Mozart's music, relative to a control group that heard a discussion of scientific experiments⁹.

Mozart's effect on mood has been verified in standard questionnaires¹⁰. In a meta-analysis of eight comparisons (with 201 subjects) of auditory relaxation instructions with Mozart's music (Table 1, bottom), the music effect appears to be larger: $d=0.20$ overall, and $d=0.56$ for spatial-temporal processing. But as relaxation instructions aim to reduce arousal, it is not surprising that they should impair subsequent cognitive performance, especially on tasks that depend on the right hemisphere.

I conclude that a shared right-hemisphere locus provides a plausible explanation for an intermittent, small positive 'enjoyment arousal' effect of Mozart's music on difficult spatial tasks. It also explains the failure to find an effect from other stimulation, which may not be sufficiently enjoyable or arousing to subjects,

Table 1 Studies of the effect of Mozart's music on the performance of cognitive tasks

Task	N	d	P
Comparisons with silence			
Raven's Advanced Progressive Matrices ¹¹	78	-0.065	0.778
Raven's Advanced Progressive Matrices ^{12*}	20	0.00	1.000
Matrices (Stanford-Binet) ^{13*}	8	0.097	0.909
Matrices (Stanford-Binet) ^{13*}	12	-0.048	0.941
Matrices (Stanford-Binet) ^{14*}	16	-0.308	0.574
Backwards digit span ¹⁵	24	0.149	0.730
Paper Folding and Cutting (Stanford-Binet) ^{1,3*}	8	1.389	0.140
Paper Folding and Cutting (Stanford-Binet) ¹⁶	136	0.218	0.209
Paper Folding and Cutting (Stanford-Binet) ^{13*}	12	-0.356	0.586
Paper Folding and Cutting (Stanford-Binet) ^{14*}	45	0.017	0.956
Paper Folding and Cutting (Stanford-Binet) ^{14*}	16	-0.989	0.085
Paper Folding and Cutting (Stanford-Binet elaborated) ^{17*}	53	0.724	0.013
Paper Folding and Cutting (Stanford-Binet elaborated) ^{17*}	38	-0.151	0.653
Paper Folding and Cutting (Stanford-Binet elaborated) ¹⁸	86	0.057	0.795
Paper Folding and Cutting (Stanford-Binet elaborated) ¹⁴	35	-0.011	0.975
Paper Folding and Cutting (computerized) ^{19*}	28	0.272	0.494
Revised Minnesota Paper Form Board ¹⁶	51	0.082	0.775
Maze completion (paper-and-pencil) ^{19*}	14	0.000	1.000
Pattern Analysis (Stanford-Binet) ^{13*}	8	0.289	0.735
Short-term memory (character strings) ^{14*}	26	-0.072	0.861
Comparisons with auditory relaxation instructions			
Raven's Advanced Progressive Matrices ¹¹	77	-0.176	0.447
Matrices (Stanford-Binet) ^{13*}	8	0.000	1.000
Paper Folding and Cutting (Stanford-Binet) ^{1,3*}	8	1.622	0.094
Paper Folding and Cutting (Stanford-Binet elaborated) ¹⁴	36	-0.032	0.925
Paper Folding and Cutting (Stanford-Binet elaborated) ^{20*}	8	0.489	0.571
Paper Folding and Cutting (Stanford-Binet elaborated) ^{21*}	32	0.814	0.033
Paper Folding and Cutting (Stanford-Binet elaborated) ^{22*}	24	0.867	0.054
Pattern Analysis (Stanford-Binet) ^{1,3*}	8	-0.685	0.434

N is the number of subjects in the comparison; d is the effect size measure, defined as the number of standard deviations by which the Mozart group performance mean is greater than the control group mean, based on the pooled variance of the two groups (performance for most tasks is measured by the number of test items correctly completed in a fixed time); and P is the two-tailed probability associated with affirming the null hypothesis (of $d=0$). When effect sizes were combined in the meta-analysis, each was weighted by its associated degrees of freedom, or $N-2$; when probabilities were combined, they were first converted to Z-values². To be included, a study must have been published or submitted for publication, and had to compare the effects on the task of a Mozart composition (usually his sonata for two pianos, K.448) and either silence or auditory relaxation instructions, not different types of music or other non-musical auditory stimuli, any of which could be the source of the effect rather than Mozart¹⁵. To ensure that none of the measurements could have been contaminated by prior tasks or listening conditions, all comparisons are between two separate groups of adult human subjects, each performing the first cognitive task in a testing session, one listening to Mozart and the other to silence or relaxation instructions of equal duration beforehand. *Extra information needed for these computations was obtained from the authors.

or on abstract reasoning or other cognitive abilities, which do not depend critically on those brain areas.

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1. Rauscher, F. H., Shaw, G. L. & Ky, K. N. *Nature* **365**, 611 (1993).
2. Rosenthal, R. *Meta-Analytic Procedures for Social Research* (Sage, Newbury Park, 1991).
3. Rauscher, F. H. & Shaw, G. L. *Percept. Motor Skills* **86**, 835–841 (1998).
4. Rauscher, F. H., Shaw, G. L. & Ky, K. N. *Neurosci. Lett.* **185**, 44–47 (1995).
5. Dittunno, P. L. & Mann, V. A. *Cortex* **26**, 177–188 (1990).
6. Heller, W. & Nitschke, J. B. *Cogn. Emot.* **11**, 637–661 (1997).
7. Robertson, I. H., Mattingley, J. B., Rorden, C. & Driver, J. *Nature* **395**, 169–172 (1998).
8. Nantais, K. M. & Schellenberg, E. G. *Psychol. Sci.* **10**, 370–373 (1999).
9. Hallam, S. *Psychol. Music* (submitted).
10. Steele, K. M., Bass, K. E. & Crook, M. D. *Psychol. Sci.* **10**, 366–369 (1999).
11. Newman, J. et al. *Percept. Motor Skills* **81**, 1379–1387 (1995).
12. Stough, C., Kerkin, B., Bates, T. & Mangan, G. *Personal. Individ. Diff.* **17**, 695 (1994).
13. Kenealy, P. & Monsef, A. *Psychologist* **7**, 346 (1994).
14. Steele, K. M. et al. *Nature* **400**, 827 (1999).
15. Steele, K. M., Ball, T. N. & Runk, R. *Percept. Motor Skills* **84**, 1179–1184 (1997).
16. Steele, K. M., Brown, J. D. & Stoeker, J. A. *Percept. Motor Skills* **88**, 843–848 (1999).
17. Carstens, C. B. & De Fruscio, D. M. *Percept. Motor Skills* (submitted).
18. Carstens, C. B., Huskins, E. H. & Hounshell, G. W. *Psychol. Rep.* **77**, 111–114 (1995).
19. Wilson, T. L. & Brown, T. L. *J. Psychol.* **131**, 365–370 (1997).
20. Rideout, B. E. & Laubach, C. M. *Percept. Motor Skills* **82**, 427–432 (1996).
21. Rideout, B. E. & Taylor, J. *Percept. Motor Skills* **85**, 112–114 (1997).
22. Rideout, B. E., Dougherty, S. & Wernert, L. *Percept. Motor Skills* **86**, 512–514 (1998).

Rauscher *et al.* reported¹ that brief exposure to a Mozart piano sonata produces a temporary increase in spatial reasoning scores, amounting to the equivalent of 8–9 IQ points on the Stanford–Binet IQ scale². Early attempts to confirm this ‘Mozart effect’ were unsuccessful^{3–6}. Rauscher *et al.* subsequently restricted their account to an improvement in spatial–temporal reasoning, as measured by the Paper Folding and Cutting task⁷. We use procedures modelled on the original report to show that there is little evidence for a direct effect of music exposure on reasoning ability.

We tested the performance of subjects on the same task (a 16- or 18-item paper folding and cutting task) after listening to the same Mozart music as in the original experiment. Control conditions were either the same or chosen to broaden the comparison set, and consisted of silence, relaxation instructions, minimalist music (*Music with Changing Parts* by P. Glass) or relaxation music (*The Shining Ones* by P. Thorton). The experimental designs replicated the original study at the University of Montreal (UM); other standard designs were used at the Appalachian State University (ASU) and the University of Western Ontario (UWO).

Table 1 shows the results of the experiments in either Stanford–Binet standard age scores (SAS) or as raw scores when con-

Table 1 Effect of listening condition on scores from the Paper Folding and Cutting task

Listening condition	Mean	s.e.	N
UM, Stanford–Binet SAS scores			
Mozart	57.3	1.26	32
Silence	59.06	0.88	32
UWO, Stanford–Binet SAS scores			
Mozart	55.58	0.64	24
Silence	54.2	0.67	21
Relaxation music	54.14	0.31	22
ASU, number correct from 16 items			
Mozart	10.78	0.74	18
Silence	11.42	0.91	17
Minimalist music	10.83	0.78	18
10 min relaxation	10.89	0.86	18
20 min relaxation	11.07	0.98	15

In the Paper Folding and Cutting (PFC) task, the subject chooses the appearance of unfolded paper from five alternatives. At UM, subjects listened to music or silence and were then given the Stanford–Binet PFC or Matrices task. After 10 min rest, they had the other treatment and were given the other task. Task order and treatment order were counterbalanced across subjects. Only results from the PFC task are shown; there was no significant effect of treatment on Matrices results, $t(30)=0.40$, $P=0.69$. At UWO, after being randomly assigned a listening condition, subjects were tested with the Stanford–Binet PFC task. At ASU, subjects were pretested with 16 PFC items. After 48 h, they were exposed to a treatment condition and tested with 16 new PFC items, followed by a 20-item mood questionnaire. PFC tasks were counterbalanced across subjects. Pretest results indicated no pre-existing difference among groups, $F(4, 81)=0.66$, $P=0.62$. There was a significant ‘practice’ effect of improvement from the first to the second test, $F(1, 81)=33.6$, $P<0.001$, but this did not interact with treatment condition, $F(4, 81)=0.59$, $P=0.67$, indicating that no treatment had a differential effect on improvement.

version was not appropriate. SAS values in the UM and UWO studies are quite similar to the original report, indicating that the subjects had similar intellectual skills. The results show that listening to the Mozart sonata produced no differential improvement in spatial reasoning in any experiment. The sonata had no effect on performance, as revealed by analyses for main effects (ASU, $F(4, 81)=0.33$, $P=0.86$; UM, $t(30)=1.14$, $P=0.263$; UWO, $F(2, 64)=1.99$, $P=0.145$) and several interactions, and for individual improvement from the pretest (ASU, $F(4, 80)=0.24$, $P=0.91$). When SAS scores were translated into IQ-point equivalents, listening to Mozart produced a 3-point increase relative to silence in one experiment (UWO, 111 versus 108) and a 4-point decrease in the other experiment (UM, 114 versus 118). Conversion of the Mozart and silence comparisons into a measure of effect size indicated that the music had little impact (mean $d=0.003$). A requiem may therefore be in order.

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1. Rauscher, F. H., Shaw, G. L. & Ky, K. N. *Nature* **365**, 611 (1993).
2. Thorndike, R. L., Hagen, E. P. & Sattler, J. M. *The Stanford–Binet Intelligence Scale*, 4th edn (Riverside, Chicago, 1986).
3. Carstens, C. B., Huskins, E. & Hounshell, G. W. *Psychol. Rep.* **77**, 111–114 (1995).
4. Kenealy, P. & Monsef, A. *Psychologist* **7**, 346 (1994).
5. Newman, J. et al. *Percept. Motor Skills* **81**, 1379–1387 (1995).
6. Stough, C., Kerkin, B., Bates, T. & Mangan, G. *Personal. Individ. Diff.* **17**, 695 (1994).
7. Rauscher, F. H. & Shaw, G. L. *Percept. Motor Skills* **86**, 835–841 (1998).

Rauscher replies — Our results on the effects of listening to Mozart’s *Sonata for Two Pianos in D Major K. 448* on spatial–temporal task performance^{1–3} have generated much interest but several misconceptions, many of which are reflected in attempts to replicate the research. The comments by Chabris and Steele *et al.* echo the most common of these: that listening to Mozart enhances intelligence. We made no such claim. The effect is limited to spatial–temporal tasks involving mental imagery and temporal ordering.

Chabris’ oversight led him to include in his analysis abstract reasoning tasks other than spatial–temporal tasks, which are a subset of the former. Four other studies (refs 4, 5 and F.H.R. *et al.*, manuscripts in preparation) all demonstrate a Mozart effect, and Chabris has excluded comparisons of scores following the playing of Mozart with scores obtained with other composers^{2,4,6,7}. The effect works for not just one spatial–temporal task, as claimed by Chabris, but for three (refs 5, 8 and F.H.R. and L. J. Hayes, manuscript in preparation). Chabris attributes our account of the Mozart effect to IQ-test variation, a fair hypothesis if the Mozart effect had anything to do with overall IQ. Test and retest reliability of spatial–temporal scores must be significantly smaller than that of general IQ score, which represents a composite of many unrelated subtests.

Chabris dismisses the neural model⁹ that motivated the original report¹ by proposing that Mozart produces ‘enjoyment arousal’, a right-hemisphere function like spatial–temporal task performance. Other abstract reasoning tasks (Ravens Matrices) are left-hemisphere functions, Chabris claims. Music therefore improves spatial–temporal tasks, not matrix tasks, as a result of a shared right-hemisphere locus. But listening to music also includes processing rhythmic information, for example, which is a left-hemisphere function¹⁰. Chabris’ reasoning would thus predict that music improves left-hemisphere tasks, such as Ravens Matrices, because of a shared left-hemisphere locus, which it does not.

Several studies suggest that the ‘enjoyment arousal’ explanation is unlikely. First,

rats exposed to the Mozart sonata *in utero* and for 60 days post-partum during their waking cycles learned a spatial maze faster and with fewer errors over days than did controls¹¹. It is unlikely that learning improved in these animals as a result of pleasure they derived from the treatment. Second, students who listened to Mozart, Mendelssohn, relaxation instructions or silence demonstrated a Mozart effect despite ratings of the Mendelssohn work as being maximally arousing⁴. Third, students who listened to the Mozart sonata scored higher on a spatial-temporal task than after they listened to other stimuli, regardless of their preference (F.H.R. *et al.*, manuscript in preparation). Finally, investigation of the Mozart effect on epileptiform activity showed that the sonata produced a reversal of the epileptic state in comatose patients¹². No effects were found after exposure to control music. According to these authors, this finding strongly suggests that the effect is not caused by emotional state or arousal.

Steele *et al.* find no Mozart effect in three differently designed studies. Not one design replicated the original reports¹⁻³, and they introduced several methodological concerns. For example, spatial-temporal task performance varies widely between individuals, making randomization an inefficient way to ensure uniform before-treatment task proficiency². What measures were taken by the two studies using between-subjects designs to tackle this? Was testing done blind, as in other replications (refs 1,3,4 and F.H.R. *et al.*, manuscript in preparation)?

Chabris' analysis is incomplete and includes studies not relevant to the effect he was exploring. Although the Mozart effect cannot be found under all laboratory conditions, as discussed by Steele *et al.*, several studies have successfully replicated it (refs 1-8,11,13,14 and F.H.R. *et al.*, manuscripts in preparation). Because some people cannot get bread to rise does not negate the existence of a 'yeast effect'.

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1. Rauscher, F. H., Shaw, G. L. & Ky, K. N. *Nature* **365**, 611 (1993).
2. Rauscher, F. H., Shaw, G. L. & Ky, K. N. *Neurosci. Lett.* **185**, 44-47 (1995).
3. Rauscher, F. H. & Shaw, G. L. *Percept. Motor Skills* **86**, 835-841 (1998).
4. Rauscher, F. H. & Ribar, R. J. *Percept. Motor Skills* (submitted).
5. Siegel, S. *Percept. Motor Skills* (submitted).
6. Rideout, B. E., Dougherty, S. & Wernert, L. *Percept. Motor Skills* **86**, 512-514 (1998).
7. Nantais, K. M. & Schellenberg, E. G. *Psychol. Sci.* **10**, 370-373 (1999).
8. Wilson, T. L. & Brown, T. L. *J. Psychol.* **131**, 365-370 (1997).
9. Leng, X. & Shaw, G. L. *Concepts Neurosci.* **2**, 229-258 (1991).
10. Peretz, I. *Brain* **113**, 1185-1205 (1990).
11. Rauscher, F. H., Robinson, K. D. & Jens, J. J. *Neurol. Res.* **20**, 427-432 (1998).
12. Hughes, J. R., Daaboul, Y., Fino, J. J. & Shaw, G. L. *Clin. Electroencephalogr.* **29**, 109-119 (1998).
13. Rideout, B. E. & Laubach, C. M. *Percept. Motor Skills* **82**, 427-432 (1996).
14. Rideout, B. E. & Taylor, J. *Percept. Motor Skills* **85**, 112-114 (1997).

Ubiquitous dispersal of microbial species

The biosphere supports astronomical numbers of free-living microorganisms that belong to an indeterminate number of species. One view¹⁻³ is that the abundance of microorganisms drives their dispersal, making them ubiquitous and resulting in a moderate global richness of species. But ubiquity is hard to demonstrate, not only because active species have a rapid turnover, but also because most species in a habitat at any moment in time are relatively rare or in some cryptic state⁴. Here we use microbes that leave traces of their recent population growth in the form of siliceous scale structures to show that all species in the chrysomonad flagellate genus *Paraphysomonas* are probably ubiquitous.

Paraphysomonas consists of 50 species, which can be distinguished by the morphology of their surface scales⁵, although oligonucleotide sequence (small-subunit ribosomal RNA) data indicate that the morphospecies are also genetically distinct⁶. The scales remain recognizable for several months after cell death, so looking at their remains in the sediment of a pond provides evidence of the preceding species succession. We used transmission electron microscopy to examine the superficial ~2 mm of sediment collected from a one-hectare freshwater pond (Priest Pot, Cumbria, UK). ²¹⁰Pb dating indicated that this sediment layer had been deposited within the previous three months. We identified and quantified all the scales and cell remains of *Paraphysomonas* species present, and used this information to reconstruct whole cells. Our examination of 25.2 µl of sediment yielded data on the relative abundance of 32 species.

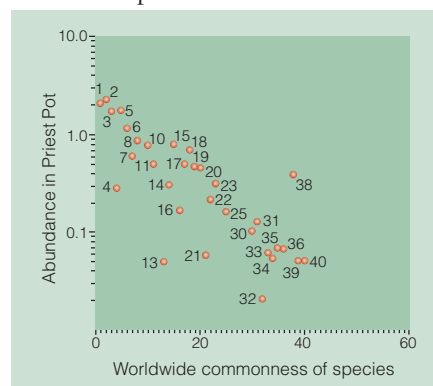


Figure 1 The abundance of each *Paraphysomonas* species in 25.2 µl (equivalent to ~0.1 cm²) of superficial Priest Pot sediment, plotted against its worldwide commonness. Commonness data are ranked in order, decreasing from left to right. Species 1 and 2 are *P. vestita* and *P. imperforata*, respectively. Further details are available from the authors.

We compared our data with information in 73 published surveys of *Paraphysomonas* species from biogeographic regions across the world. These surveys recorded a total of 41 species, 78% of which were detected in our small volume of pond sediment. The pattern of relative abundance of species in Priest Pot is similar to the global one (Fig. 1). Species that are frequently recorded globally are also abundant in sediment from Priest Pot, and species that are rarely found globally are not abundant in Priest Pot.

We think that globally abundant species will, through neutral migration, 'seed' the pond more frequently than rare species. They are probably capable of population growth in a broad range of conditions, so they will more frequently find suitable conditions. Finally, termination of population growth is accompanied by the production of resting cysts. As the size of the 'cyst bank' for each species is likely to be proportional to its global abundance, repeated cyst production will effectively strengthen the pattern of relative abundance of species that results from neutral migration.

It is widely believed that most microbial species have yet to be discovered, which follows from the general rule that, for each tenfold reduction in body length, the global number of taxa increases roughly 100-fold⁸. But this relation breaks down for organisms smaller than about 1 mm (refs 1,8,9), probably because the enormous number of microorganisms (the water column of Priest Pot typically supports ~4 × 10¹⁴ living *Paraphysomonas*) drives large-scale dispersal across the physical and geographical barriers that halt the migrations of larger animals and plants. As ubiquity will limit rates of local speciation and extinction¹, the global number of species less than 1 mm long will be relatively small.

Free-living bacteria sustain all the important ecosystem functions. They are about three orders of magnitude more numerous than heterotrophic flagellates, so it is even more likely that they too are ubiquitous, and that the global richness of free-living microbial species is moderate.

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1. Fenchel, T. *Oikos* **68**, 375-378 (1993).
2. Roberts, M. S. & Cohan, F. M. *Evolution* **49**, 1081-1094 (1995).
3. Finlay, B. J., Esteban, G. F. & Fenchel, T. *Nature* **383**, 132-133 (1996).
4. Fenchel, T., Esteban, G. F. & Finlay, B. J. *Oikos* **80**, 220-225 (1997).
5. Preisig, H. R., Vörns, N. & Hållfors, G. in *The Biology of Free-living Heterotrophic Flagellates* (eds Patterson, D. J. & Larsen, I.) 361-399 (Syst. Assoc. Special Volume No. 45; Clarendon, Oxford, 1991).
6. Rice, J. *et al. Microbiology* **143**, 1717-1727 (1997).
7. Finlay, B. J., Maberly, S. C. & Cooper, J. I. *Oikos* **80**, 209-213 (1997).
8. May, R. M. *Phil. Trans. R. Soc. Lond. B* **330**, 293-304 (1990).
9. Lawton, J. H. *Oikos* **81**, 3-5 (1998).

The universal structure of babbling

How Language Comes to Children

by Bénédicte de Boysson-Bardies

MIT Press: 1999. 257 pp. \$27.50, £19.50

Massimo Piattelli-Palmarini

The ideas of famous authors, no matter how subtle, rich and complex, often circulate condensed in very short sentences. From Noam Chomsky, who tops, among the living, the sheer number of quotations in other authors' works, we have two such sentences here. They have been received, in the course of almost five decades, with admiring consent by some linguists, with disgust by others, and with perplexity by many non-linguists. The first is: "Learning our mother language is not something that we do; it's something that happens to us." The second says: "The scientific value of the expression 'learning one's mother language' is the same as that of the expression: 'The sun rises in the morning'. That is: zero!"

The title of a book by Bénédicte de Boysson-Bardies on language acquisition, *How Language Comes to Children*, has indeed an unmistakable Chomskyan flavour. Generative grammar is the name for the line of enquiry opened by Chomsky since the mid-1950s, and it is this line that looms large in the entire book, just as it has in the original experimental research for which the author is known. The core idea of this approach, or at any rate the thesis that has been most frequently singled out for praise or for blame, is (to put it very bluntly) that language is innate. The author's declared aim is to make the ideas, methods and results of a universalist, innatist approach to language acquisition accessible to a wider public.

Steven Pinker's masterful *The Language Instinct* (Harper, 1994), now available in several foreign translations, has quickly become a scientific best seller. I can bear witness to its success in conquering the assent of many uninitiated readers, including some who were initially hostile to Chomsky's ideas. In the domain of popularization and persuasion, therefore, Pinker has surpassed the master.

At the price of being more than a bit unfair, one cannot help comparing de Boysson-Bardies' book to Pinker's. Both stem from the same scientific roots, are similar in scope and ambition, and, sure enough, quotations from Pinker are abundant. While Pinker has painted in vivid colours a vast landscape, de Boysson-Bardies is poised to draw accurate sepia sketches of fascinating corners of that landscape. The baby's own perspective is constantly emphasized. Digging out a wealth of little-known historical antecedents, inserting pearls of quotations from a variety of unexpected sources, and



Learning by rote: but the skill of language acquisition is innate.

exploring the habits of child-rearing in distant cultures, this book manages to exhibit all the essential facts relevant to language acquisition, from the last months of gestation to birth, then to the first weeks, months and years of life. The neurological correlates of the various language stages have also been closely tracked.

De Boysson-Bardies has taken great care to untangle the universal components, common to babies the world over, from a variety of interesting specificities, proper to the different languages. For instance, the patterns of spontaneous vocalizations, characteristic of the babbling of babies reared in a strictly mono-lingual environment in France, are compared with those of American, Algerian, Cantonese and Japanese babies, who are likewise linguistically confined. Interesting analogies are, thus, revealed between the profiles of babbling and the phonological and prosodic patterns of the corresponding adult language. Contrary to a still dominant tradition, babbling is here vindicated as preparatory to, and continuous with, the profiles of the first words produced by the child many months later.

In an appendix, the author reconstructs a timetable of language development. An intelligent use of charts, lists, histograms and sketches of experimental designs helps the reader to become familiar with the core of the investigative techniques through which "the Sherlock Holmeses" of early language acquisition can ask precise questions of their little subjects, and be rewarded sometimes with loud and clear answers.

Alas, here and there, mostly when she covers her own work and that of her collaborators, de Boysson-Bardies gets carried away,

indulging in details that are inordinately minute for a book of this size and ambition. Teachers and students of psycholinguistics, developmental psychology and infant cognition may well treasure all such details, but not every reader can be expected to enjoy them. True enough, behind our close encounters with endearing Léo, Emilie, Sean, Marc, Timmy, Marie and their mothers, there is always some big, universal question. For instance: are the systematically distorted words that the child produces stored in memory as being the same as the adult's words that the child perceives and recognizes? How general are the child's linguistic learning strategies? Is it really the case that, to a cold scientific eye, learning English is exactly the same as learning Cantonese?

Chomsky likes to suggest that, to an intelligent Martian, all human languages and dialects look structurally the same, in spite of certifiable superficial differences. This remark makes linguists of a different persuasion draw their revolvers. De Boysson-Bardies is determined to have a closer look at real data from cross-linguistic developmental studies, charting more precise frontiers of sameness and difference between languages than the Martian would. She is adamant in arguing that, in this domain, clear-cut and simple answers are mostly untrue. We are, therefore, duly exposed to subtle differences between languages, and between individual strategies of language acquisition within the same language.

Inside the genetically determined envelope of what is linguistically possible, the child has leeway to choose his or her personal avenue to the mother tongue. In the author's own words: "Children's styles or modes of

accessing language show themselves to be incredibly different. How can this be explained on the basis of common mechanisms?" Two-hundred-odd pages of clear prose built on an enviable expertise make it very clear that this is not a rhetorical question. Boysson-Bardies' snapshots of language acquisition are all taken from Mount Universal. The core message is simple: only when looking down from that peak can we really follow the fine interweaving of the innate and the acquired components of the child's linguistic capabilities. □

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Thou shalt not mix religion and science

Rocks of Ages: Science and Religion in the Fullness of Life

by Stephen Jay Gould

Ballantine: 1999. 241 pp. \$18.95

S. Nomanul Haq

"I am inclined to look at everything as resulting from designed laws, with the details, whether good or bad, left to the working out of what we may call chance," Charles Darwin is quoted as having said in Gould's impassioned thesis on the age-old question of science and religion. It is in this remark that the kernel of Gould's thesis lies. There is no essential conflict between science and religion — in the fullness of life they are both vital, but they belong to two utterly different realms which do not overlap and cannot be synthesized.

Quite remarkably, this thesis is expressed and elaborated on in this petite volume in a highly personalized, colloquial parlance. It moves far and wide in the process — into the history of science, politics, biological evolution, and even classical poetry. He moves back and forth between the nature and scope of science, to the essence of religion. This is an ambitious synthesis, but it neither intimidates nor does it seem too far-fetched. Gould appeals to common sense, and deliberately sticks to that level.

Gould tells us that the "details" referred to by Darwin in the above quote are precisely and exhaustively the business of science. Details here mean empirical facts, in contrast to larger questions concerning the ultimate drift and meaning of the cosmos. And going beyond Darwin's remark, Gould adds that these facts exist for *immediate*, not transcendental reasons: reasons that are knowable through rational means and subject to scientific explanation. There was no "intended meaning in the fall of each petal and every raindrop" — this is what Darwin meant by "chance"; it meant "contingency" as



The battle between creationists and scientists in the US courts is not part of a wider war between science and religion, argues Gould. Above: anti-evolution books on sale during the Scopes trial of 1925.

opposed to some pre-ordained design. Science is a factual construction of the world, involving the development of theories that coordinate and explain nature's empirical data. Nature exists in "sublime indifference" to *Homo sapiens*, with "no preference for accommodating our yearnings", no matter how much we long, like the Persian poet Omar Khayyam, whom Gould quotes, to mould it to our shape.

But factual questions about the world, Gould explains, define both the scope as well as the limitation of science — its magisterium. Science has no business making moral pronouncements; factual truth cannot dictate moral truth. Questions of meaning and morals, of life's ultimate purpose and values, of human fellowship and ethical conduct — these belong properly to the institution called 'religion', embodying a different magisterium.

Just as science has its limitations, so has religion. If life's evolutionary history cannot resolve the riddle of life's meaning, so the religious belief concerning the creation of the world in six days, taken literally, cannot dictate or interfere with factual conclusions in the empirical realm of cosmology. Science and religion, then, embody two logically distinct magisteria, each having its own style of enquiry, its own set of standards and norms, and its own test of legitimacy. Neither of them encompasses all enquiry. Into this framework comes Gould's core declaration: science and religion embody two equally important but utterly different Non-Overlapping Magisteria, or NOMA.

Gould's NOMA, which is often reminiscent of Thomas Kuhn's paradigm, is at once a prescriptive principle and a historical representation. On the one hand, Gould says that scientists and believers ought not to violate the principle of NOMA. This would allow

the two magisteria to coexist and flourish in a position of respectful non-interference, with one seeking inspiration and illumination from the other without the two fusing. To paraphrase: as scientists, we look into the palpable reality of the natural world; as believers, we look into our inner beings. In this way we fashion a quilt of understanding with distinct, non-overlapping patches — and we call this patchwork wisdom.

On the other hand, articulating some of the latest historical research, Gould claims that the principle of NOMA has been effectively respected throughout the history of science. In truth, there was no conflict between science and religion; the conflict existed only in people's minds, not in historical reality. Thus, on the authority of a contemporary historian, Gould points out that the famous trial and forced recantation of Galileo in 1633 was a political drama of a princely court, not a science-versus-religion fight. Similarly, on compelling historical grounds, Gould challenges what he considers a legend: that medieval church fathers in general taught the doctrine of a flat Earth, and that Columbus suffered in the hands of ecclesiastical authorities over this "non-issue".

Finally, recalling in detail the fierce and scandalous creationism versus evolution battle in the American courts, a battle in which Gould himself fought, and which has been recently revived, he tells us once again that this was a political phenomenon of a uniquely American kind, not science against religion.

It is hard to imagine reasonable minds having any quarrels with Gould's motives: deeply concerned about science, he wants to protect it from the attacks of naive and misguided religious zealots who understand neither science nor religion. At the same time, he wishes to instill confidence in scientific circles that human history is on their

side, that religion in its essence is not an adversary, and that the persecution or rejection of scientists in the name of religion has always been rooted in non-religious reasons.

Here is Gould, an accomplished scientist, breaking what I call the 'contrariety myth' — that religion and science cannot subsist together and that one must feed on the arterial blood of the other. Here, a scientist, who introduces himself as an agnostic, pays tribute to religion, considering it to be vital in the fullness of life, and rehabilitating the believer in a position of utmost respect. Gould's motives are so glorious and his concerns so timely that one is tempted to support him even if his analysis turns out to be problematic in its "details".

Speaking of details, scholars of religion may raise the objection, for example, that the scientist Gould inflates the status of religion only to puncture it because he places fundamental limitations on God — and admits it. Fashion your God freely, he says, but do not attribute to Him the power to work miracles. But is this a theological position — or is it a violation of NOMA? Moreover, do we really think that believers will ever truncate their God in this way? Would it not destroy the very foundation of the concept of Godhead? Scholars may also object that Gould's analysis is too narrowly restricted to the Judeo-Christian European milieu — what about Arabic science, which is an integral part of the history of the discipline as we know it? The secular-religious dichotomy blurs in the case of Islam, but then it had no 'Inquisition' and no official persecution of scientists took place: how do we explain this? And what then becomes of Gould's thesis?

Professional philosophers and historians of science may accuse Gould of stepping on their toes. They may say that his account of "facts" as something independent of a theoretical or metaphysical framework is oversimplified, that his neat distinction between the ethical and the factual is problematic. While historians may share his impatience with any contemporary agendas fusing science and

religion, his logical thesis of the incommensurability of the two is not quite borne out by historical data, as he should be aware.

But Gould still comes out as the winner. He has not written this work for specialists; it is aimed rather at the general reader. It operates on a common-sense level and, ultimately, it makes sense. So, whatever issues one may legitimately have with its 'details', in its general thrust this small book is heftier than the volumes of inaccessible, jargon-wrapped material produced every year by some academic historians and philosophers. *Rocks of Ages* should be read not only in all our colleges, it should be read in our temples and mosques, in our churches and synagogues. □

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Dogged footsteps to a vanishing past

Saddled with Darwin: A Journey Through South America

by Toby Green

Weidenfeld & Nicolson: 1999. 366 pp. £20

Andrew Berry

Darwin's visit to the Galapagos Islands during his voyage on the *Beagle* did not, as is popularly supposed, supply him with a Eureka! moment. It was his careful and wide-ranging observations on the geology and natural history of the Galapagos and many other ports of call that formed the intellectual foundation on which — after 25 years of gestation and synthesis — the *Origin* was built. The journey, however, had another long-term impact on Darwin.

By providing him with the ultimate in exotic travel experiences — the "savages" of Tierra del Fuego, an earthquake in Valdivia, Chile, a first-hand encounter with that most bizarre of animals, the platypus — the voyage seems to have sucked a lifetime's worth of wanderlust out of him. He never again left Britain, and, within five years of his return, had moved into Down House and settled into the sedentary existence that characterized the remaining 40 years of his life.

One of many reasons for this abrupt shift from adventurer to reclusive family man may have been the misery associated for Darwin with ocean travel. He was so hopelessly seasick that Captain Fitzroy considered off-loading him at the first stop at the Cape Verde Islands. "I hate every wave of the ocean," Darwin wrote to his cousin, William Darwin Fox, "with a fervour which you who have only seen the green waters of the shore can never understand." One solution to this problem was to forsake the *Beagle* for dry land whenever possible. As a result, Darwin spent less than 18 months of the entire five-year journey at sea, choosing to undertake

lengthy trips inland over the alternative of staying with the ship as it went about its surveying work.

In *Saddled with Darwin*, Toby Green tries to retrace Darwin's footsteps through South America, both on horseback and on foot. This proved a frustrating exercise. Inevitably, there are sections of the route that are no longer accessible: for example, Darwin's road through the valley of Chile's Rio Tinguiririca is now submerged under a lake that feeds a hydroelectric project. And Green chose to make the journey even harder by adopting the peculiarly British approach to travel — pioneered by Eric Newby in his *A Short Walk in the Hindu Kush* (Lonely Planet, 1998) — of being largely unprepared for what is to come. Thus, as he sets out for an epic horseback journey of several thousand kilometres, he boasts that the "main drawback was that I could not ride a horse".

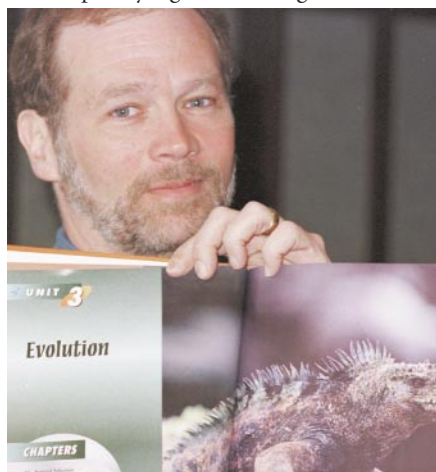
Green fails to explain why he did this. Fresh from an undergraduate degree in philosophy, he has no particular expertise on or interest in Darwin or evolutionary biology, though he does throw in occasional asides on topics such as sociobiology. Darwin was, therefore, little more than a contrived pretext for the journey: Green needed a route to follow through South America, and Darwin provided him with one.

But Green is nevertheless dogged in his pursuit of Darwin, going to extraordinary lengths to visit as many as possible of the places mentioned in the *Beagle* narrative. I found myself wishing from time to time, as he struggled through desolate country, that he had heeded the advice offered by Roberto in Buenos Aires: "Why don't you go somewhere nice and just tell everyone you followed Darwin?" The trouble with following Darwin for the sake of following Darwin is that you visit all sorts of places you would never otherwise dream of going to. What, in Darwin's day, may have been a charming provincial town has become a sprawling industrial nightmare.

Unlike Darwin, Green's interests are primarily sociological, and the book is best read as a catalogue of the extraordinary people, most of them poor and beset with problems of their own, who were hospitable to, helpful to, and/or got drunk with Green when he showed up on their doorsteps. As such, *Saddled with Darwin* is effectively a typical modern travelogue, in the tradition of Bruce Chatwin or Paul Theroux, rather than part of the burgeoning Darwin literature.

Green's account of the people he met on the way is a delight. He is at his best when sharing a few companionable drinks — whether maté tea, wine, *chicha* (alcoholic cider), or *pisco* — with the likes of Ramon, "wild man and then a repentant Mormon missionary", marooned in the wilds of Navarino Island close to Cape Horn.

In the end, Green does himself a disservice in harnessing himself to Darwin because this inevitably invites comparison — perhaps an



Defending the study of evolution today: textbook author Ken Miller campaigns on the issue.

CHRIS PPUHL/AP

unfair one. Darwin's spare, analytical prose contrasts strongly with Green's, which is marked by overblown descriptive passages. Viewing the Andes from La Campana, a hill inland from Valparaiso, Darwin admires "the wonderful force which has upheaved these mountains," while Green observes that "the Andes rose from [the valleys] as a phoenix, laughing maniacally at the ashes below".

Readers hoping for insights into the workings of the young Darwin's mind, or for a sense of on-the-road kinship with Darwin, will be disappointed. But, as a straight travel book, Green's has many charms, though his superficial preoccupation with Darwin both distracts and detracts from its account of an extraordinary journey. *Saddled with Darwin* does indeed seem uncomfortably (and unnecessarily) saddled with Darwin. □

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Mainstreaming monsters

Wonders and the Order of Nature 1150–1750

by Lorraine Daston and Katharine Park
Zone Books: 1998. 511 pp. \$35.95, £22.50

The Ambonese Curiosity Cabinet

by Georgius Everhardus Rumphius
Yale University Press: 1999. 567 pp. \$45, £30

Harriet Ritvo

Pigs with human heads and feet may now seem the incontestable province of tabloid newspapers, but they and similarly astonishing violations of the quotidian order played an important role in shaping the thought of natural philosophers and natural historians of the medieval period, the Renaissance and the Enlightenment. Over the past few decades, monsters have emerged, once again, from the shadowy corners of the history of science, to bask and wriggle in the full glare of academic scrutiny. No longer stigmatized as mere antiquarian oddities, they have taken their place among more traditional topics of scholarly investigation.

This signals a larger alteration in our understanding of the scientific past. The objects of elite investigation and vulgar curiosity have ceased to appear so widely separated, and we no longer focus on the antecedents of current orthodoxy to the exclusion of ideas and methods that may have seemed equally plausible, or even more so, at the time when they were formulated.

In *Wonders and the Order of Nature, 1150–1750*, Lorraine Daston and Katherine Park assert the intellectual importance of wonder, the now-discredited emotion that such inexplicable phenomena often inspired in scholars, as well as in relatively naïve



Boo: the Krakow monster of the 1540s portrayed like a demon, with heads on its joints.

observers. They emphasize the extent to which not only modern attitudes, but also the categories that we automatically deploy, such as the dichotomy between the natural and the supernatural, or between the natural and the artificial, may obscure our perception of earlier world views.

They magisterially survey developments in the content and function of wonder over a period of six centuries, stretching from the high Middle Ages to the Enlightenment, at which point wonder was, they argue, decisively banished from the intellectual arsenal of respectable European science. Monsters are not the only subjects of this handsomely designed and generously illustrated volume, but they offer the most compelling and vivid examples of its larger concerns.

But the trajectory the authors delineate was far from straightforward. The fortunes of wonder, and of the marvellous objects in which it was embodied, rose and fell, as religious, intellectual and political commitments shifted. At its medieval zenith, the admiration and accumulation of marvels struck members of both the spiritual and temporal ascendancies as a way of confirming the established order. As is normally the case, however, consensus was neither absolute nor stable. Dissenting scholars associated wonder with ignorance and fear. The intellectual stock of wonder rose again with the European voyages of discovery, which revealed incontestable marvels previously undreamed of, but then fell as the religious and political turmoil of the seventeenth century reconfirmed their association with disruption and fearsome portents.

After a brief renaissance early in the Enlightenment, marvels retreated to the disreputable position that they have occupied ever since. This very brief summary can only gesture at the richness and complexity of the story that Daston and Park have to tell. Indeed, one of the most impressive features of the book is the skill and clarity with which its authors explicate subtle alterations in

points of view and philosophical positions — despite their obscurity and counter-intuitiveness to modern sensibilities.

Daston and Park present an authoritative rereading of premodern ideas about the natural order. Like any similarly ambitious project, it has limitations and omissions. The authors point out, for example, that, despite their efforts at contextualization, they have focused on elite ideas. And the ineluctable need to reach a conclusion has led them to exaggerate reports of their subject's ultimate demise.

However strong their triumphalist protestations, eighteenth-century naturalists did not definitively jettison the marvellous. The flotsam of the midway continued to fascinate and trouble scientists well into the nineteenth century, for the same reasons that anomalies had captivated their predecessors. Exceptions to the order of nature were illuminating, if they could ultimately be made to fit — and if they couldn't be made to fit, then they had to be debunked, in order to eliminate a threat that was both intellectual and social.

Georgius Everhardus Rumphius (also known as Rumpf), a cog in the elaborate Dutch colonial machinery of the seventeenth century and the author of *The Ambonese Curiosity Cabinet*, does not appear in Park and Daston's extensive bibliography of sources. Nevertheless, his elaborate catalogue — which deals primarily with marine invertebrates native to Indonesian waters, but which also describes miscellaneous other wonders, including remarkable minerals, as well as potent stony objects extracted from the innards of cats, pigs, deer and people — is representative of one large category of material upon which they draw in the second half of *Wonders and the Order of Nature*.

As such, it gives a concrete sense of the scope and difficulty of the research that underlies their argument. It also suggests why many of their primary sources, although they have been published at some point, remain inaccessible to most modern readers — doubly immured in libraries of rare books and in Latin or in obscure early versions of modern languages. At the core of the new edition of *The Ambonese Curiosity Cabinet* is a translation by the editor, E. M. Beekman, from seventeenth-century Dutch. The edition is well illustrated and includes extensive apparatus: background discussions of Rumphius's life and career, and of the special problems posed by the translation; a bibliography of his work; an elaborate general bibliography; and extensive notes. It constitutes a valuable contribution to the study of early colonial science — but it is easy to see why such editions do not exist for every equally interesting early work of natural philosophy or natural history. □

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Structure of a bacterial 30S ribosomal subunit at 5.5 Å resolution

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The 30S ribosomal subunit binds messenger RNA and the anticodon stem-loop of transfer RNA during protein synthesis. A crystallographic analysis of the structure of the subunit from the bacterium *Thermus thermophilus* is presented. At a resolution of 5.5 Å, the phosphate backbone of the ribosomal RNA is visible, as are the α -helices of the ribosomal proteins, enabling double-helical regions of RNA to be identified throughout the subunit, all seven of the small-subunit proteins of known crystal structure to be positioned in the electron density map, and the fold of the entire central domain of the small-subunit ribosomal RNA to be determined.

Translation of the genetic code on mRNA into a polypeptide chain takes place on the ribosome, a large nucleoprotein complex that consists of two subunits, one large and one small. In bacteria, the large and small ribosomal subunits are designated 50S and 30S, respectively, and together they form the 70S ribosome. Each ribosomal subunit is about two-thirds RNA by mass and consists of many different proteins. In particular, the small ribosomal subunit of *Escherichia coli* has a relative molecular mass of about 0.9×10^6 ($M_r = 900$ K) and consists of a piece of RNA 1,542 nucleotides long, designated 16S RNA, and 21 different proteins¹.

The ribosome is an intricate molecular machine that is actively involved in the various steps of protein synthesis. Peptidyltransferase activity is associated with the 50S subunit, whereas the 30S subunit provides the framework for decoding genetic information, which involves detecting that a match has been made between the anticodon of tRNA and the codon on mRNA. The 30S subunit also has a sophisticated proofreading mechanism to minimize translation errors. During elongation of the peptide chain and translocation

of the ribosome along the mRNA, there is a concerted movement of the mRNA and bound tRNA by precisely one codon relative to the ribosome. This movement must involve breaking and reformation of precise contacts, and is particularly interesting in the context of the 30S subunit because of its role in codon recognition. Both large and small ribosomal subunits are targets for several clinically relevant antibiotics.

Crystals of the 50S subunit were obtained that diffracted to beyond 3 Å (ref. 2), indicating that the determination of an atomic-resolution structure of a ribosomal subunit was possible in principle. Using a modification of earlier crystallization conditions³, we have obtained crystals of the 30S ribosomal subunit from *T. Thermophilus* that diffract to 3.6 Å resolution. These crystals are a significant improvement over those reported previously which diffracted to resolutions of 9–12 Å (refs 4, 5), but they are comparable to an improved form obtained independently⁶.

We present our current analysis of the structure of the 30S ribosomal subunit, based on an electron-density map at 5.5 Å

Table 1 Summary of crystallographic data and analysis

Data-collection statistics							
Crystal	Estimated mosaicity (deg)	Resolution limit (Å)	Completeness (%)		Redundancy	R_{sym}	
			Overall	Outer shell		Overall	Outer shell
Native	0.56	6.76	95	92	20	0.048	0.079
Os	0.33	5.26	95	83	5	0.081	0.196
Yb	0.41	6.01	97	95	5	0.080	0.223
Lu	0.58	6.50	97	90	6	0.078	0.234
Ta	0.45	7.00	99	100	5	0.098	0.278
W12Si	0.26	6.00	96	79	4	0.113	0.336
W17	0.64	6.75	96	88	4	0.099	0.251
Phasing statistics							
Derivative	Number of sites	R_{cullis} (centric)	Phasing power				
			Isomorphous	Anomalous			
Ta	3	0.73	0.48	0.67			
W12Si	7	0.72	0.51	0.72			
W17	2	0.72	0.61	0.80			
Os	23	–	–	1.4			
Lu	11	0.74	0.47	0.64			
Yb	10	0.74	0.48	0.64			

Overall mean figure of merit (before solvent flattening): 0.56

Os, osmium hexamine chloride; Yb, ytterbium chloride; Lu, lutetium chloride; Ta, Ta₆Br₁₂; W12Si, H₂SiO₄[12WO₄]; W17, Li₁₀[P₂W₁₇O₆₁].

$R_{\text{cullis}} = (\text{lack of closure}) / (F_{\text{ph}} - F_{\text{ph}})$.

Phasing power = $(|F_{\text{h}}(\text{calc})| / \text{lack of closure})$.

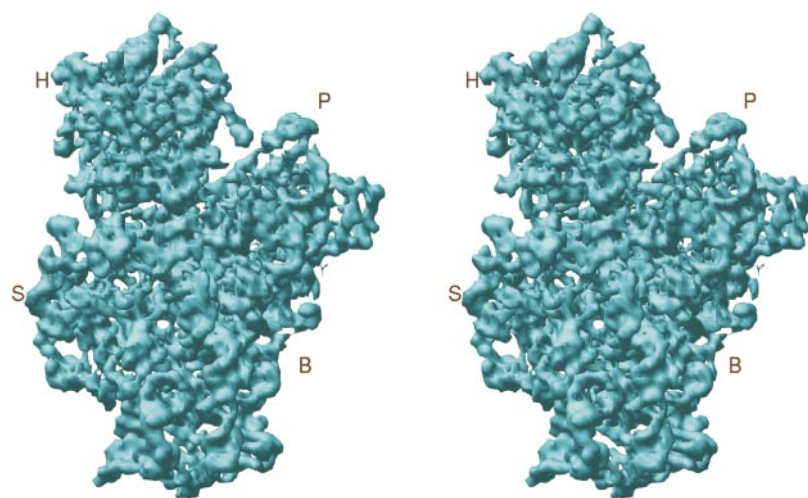


Figure 1 Electron-density map of the 30S subunit at 5.5 Å resolution. Stereo view of a surface contoured at 1 s.d. displayed using RIBBONS⁵⁰. H, head; P, platform; S, shoulder; B, body. The intersubunit interface is towards the reader.

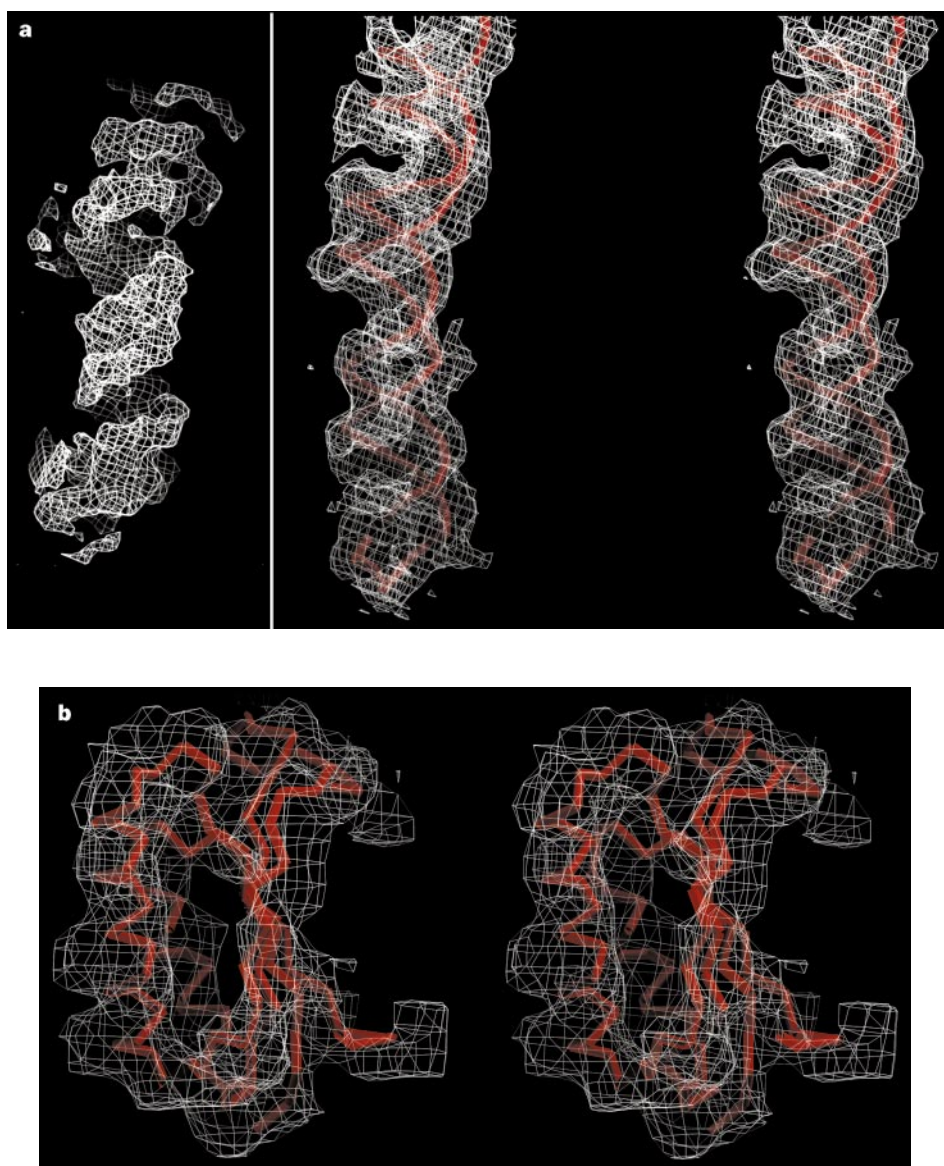
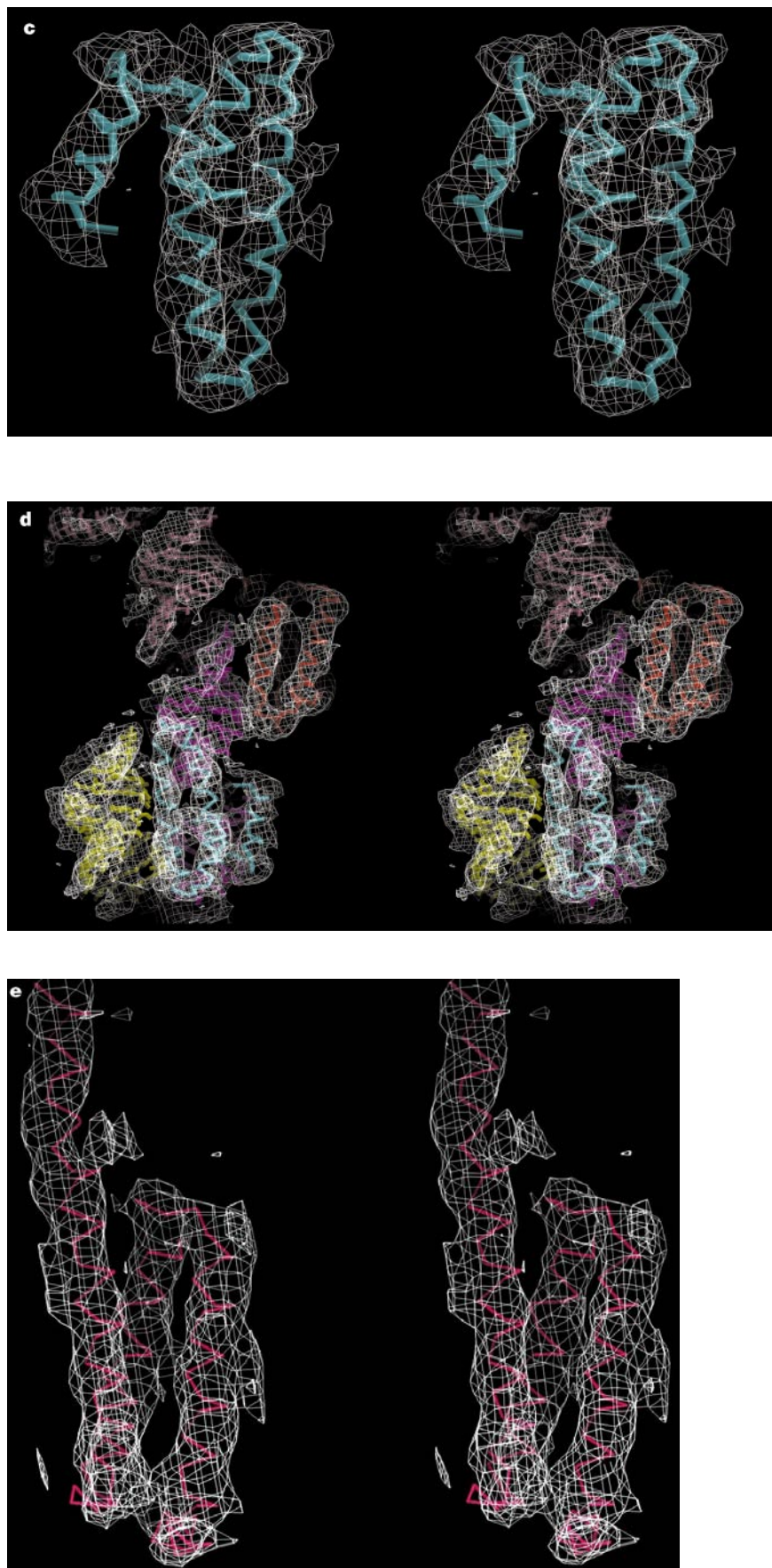


Figure 2 Features visible at 5.5 Å resolution. **a**, Double-helical RNA corresponding to the 1400/1500 stem-loop of 16S RNA. Left, density with a right-handed double-helical twist with clear major and minor grooves, individual phosphate strands and bumps at the right separation for phosphate groups. Right, stereo



view of a fit of short stretches of A-form double helix to the density. The tetraloop at the end was fitted to the structure determined by NMR¹⁰. **b, c**, Fits of proteins of known crystal structure into the electron density. The panels show stereo views

of: **b**, S6 (ref. 12); **c**, S15 (refs 15, 18). **d**, Protein-RNA complexes. The region shows S6 (red) and S15 (cyan) bound to double-helical RNA. **e**, Putative structure of S20, for which no high-resolution structure exists. Figure made using O⁴⁹.

resolution. At this resolution, double-helical RNA and α -helices and β -sheets of proteins can be identified. As a result, all seven 30S-subunit ribosomal proteins of previously known structure and a large fraction of the double-stranded RNA helices have been fitted into density. α -Helices of many of the proteins of unknown structure are also evident, as well as their interactions with neighbouring RNA. We have also determined the fold of the central domain of 16S rRNA, a region which includes the platform. This part of the 30S subunit is particularly important in its decoding, large-subunit associating and proof-reading activities. The structure helps to rationalize a large body of biochemical data and establishes a new framework for mechanistic studies of translation.

Analysis of the structure

The structure was solved using multiple isomorphous replacement including anomalous scattering. An osmium derivative was used as the 'native' or reference data set because it had the best diffraction. Crystallization, data collection and phasing were done as described (see Methods), and the crystallographic data are summarized in Table 1. The final resolution of the map is about 5.5 Å, as judged by both phasing statistics and visual inspection of the map.

Overall shape

The crystallographic asymmetric unit contains a single small subunit. As shown in Fig. 1, the small subunit in the crystal clearly exhibits the characteristic head, platform, shoulder and body

familiar from electron-microscopy studies of 30S subunits from *E. coli*⁷ or *T. Thermophilus*⁸. The dimensions of the subunit quantitatively agree with the results from these earlier electron-microscopy studies.

Features visible at 5.5 Å resolution

A long, protein-free stretch of double-helical density is visible at the subunit surface, seen running roughly vertically in Fig. 1. This long double helix is the penultimate stem-loop of 16S RNA (the 1,400/1,500 region), believed to be located at the interface between the two subunits⁹. At a resolution of 5.5 Å, the two strands of RNA are clearly distinguishable (Fig. 2a), so that the deep major and shallow minor grooves can be seen. The tetraloop at the end of this stem agrees with the conformation determined by nuclear magnetic resonance (NMR) methods¹⁰. In well ordered double-helical regions, individual phosphates appear as bumps in the density along each strand. Elsewhere in the map, single-stranded RNA is visible as tubes of backbone density joining double-stranded helices.

α -Helices of proteins appear as tubes at this resolution. Weaker density is also generally seen for β -sheets and even for well ordered loops. We can therefore fit individual proteins of known structure into the map, as shown for proteins S6 and S15 (Fig. 2b, c). Protein-RNA complexes can be identified for proteins of known structure involving double-helical RNA. An example is shown in Fig. 2d, where S6 and S15 make contact with the same stretch of double-helical RNA.

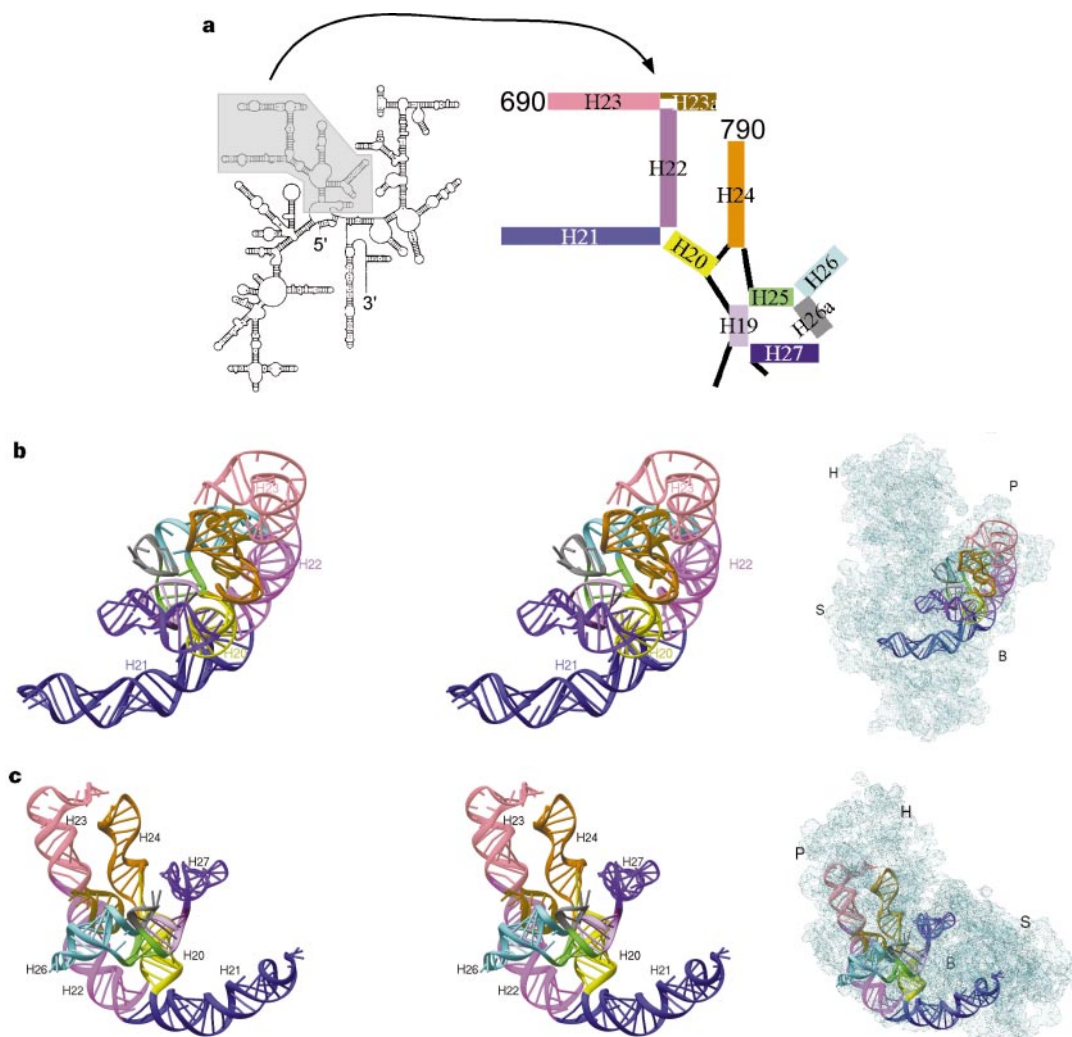


Figure 3 The central domain of 16S RNA. **a**, Left, secondary-structure diagram of 16S RNA, with central domain shaded; right, expanded view of the central domain with individual helices coloured and the 690 and 790 loops indicated. **b**, **c**, Stereo

views of the tertiary fold of the central domain, using the colouring scheme for the helices shown in **a**. Insets (right) show the corresponding views in the context of the 30S subunit. Figure made with RIBBONS⁵⁰.

Placement of proteins in the 30S subunit

We have been able to place all seven of the proteins whose high-resolution structure is known, namely S4, S5, S6, S7, S8, S15 and S17 (refs 11–21). In the absence of other information, the localization of known protein structures in such a large asymmetric unit would have been problematic even at this resolution. However, there are extensive data on the approximate location of the proteins in the 30S subunit, as well as their interactions with 16S RNA. The neutron map of the centres of mass of the 30S proteins²² particularly facilitated and accelerated the identification of density for specific proteins. Data from footprinting studies²³ and extensive biochemical data (summarized in ref. 24) also provide clues about the location of proteins in the subunit. Several of these proteins are known to be adjacent to or to bind to neighbouring regions of 16S RNA.

The placement of these seven known structures in the 5.5 Å map was compared with the neutron map by calculating a least-squares superposition of the neutron map into the observed centre-of-mass of the coordinates of the positioned proteins. When S15 is excluded from the comparison, the neutron map is in generally good agreement with the actual location observed here, with an r.m.s. deviation (r.m.s.d.) of 11.8 Å, which is comparable to the mean coordinate error in the neutron map²². However, this superposition places S15 roughly 51 Å from its position in the neutron map. The discrepancy could arise from a single distance measurement (S11–S15) in the neutron data, which has poor statistics²⁵. A definitive assessment of the overall quality of the neutron map must await the identification of the remaining proteins in the subunit.

We have also identified proteins S2, S3, S11, S16 and S18, whose

structures have not yet been determined, on the basis of α -helical density in the regions predicted for these proteins from biochemical and neutron-scattering data. At this point, their identification must be considered tentative. Near the bottom of the subunit, there is a three-helix bundle (Fig. 2e). This protein could be modelled in some detail as all of the loops can be traced in the electron density, and we have identified it as S20 on the basis of biochemical data^{23,26}. Our model for the protein represents a new protein structure, albeit at low resolution, and is consistent with secondary-structure predictions based on the amino-acid sequence. However, our location for S20, although it is consistent with both biochemical data and a previous model²⁴, is not in agreement with the neutron map, which places S20 in the head of the subunit.

The central domain of 16S RNA

The central domain of 16S RNA spans nucleotides 565–885 (*E. coli* sequence). Figure 3a shows the secondary structure of the central domain using the scheme adopted in ref. 23 and the helix numbering of ref. 27. This portion of the 16S RNA and its associated proteins form the platform and part of the body of the 30S subunit. The platform is a flexible and functionally important structure containing the 690 and 790 stem-loops, which have been identified as crucial for binding of P-site tRNA²⁸ and for subunit association^{29,30}. At the 3' end of the central domain is another functionally important region, H27, which has been proposed to function as an essential conformational switch that affects translational accuracy³¹. The central domain is also one of the biochemically best-characterized parts of 16S RNA. It contains the binding sites for ribosomal proteins S6, S8, S11, S15 and S18 (refs 23, 32, 33). In



Figure 4 Stereo view of the three-way junction formed by helices 20, 21 and 22 of the central domain of 16S RNA. Inset (right) shows the structure in the context of the 30S subunit. Figure made with RIBBONS⁵⁰.



Figure 5 Stereo view of the interactions made by helix 27 of the central domain with helices 24 and 44 of 16S RNA. Inset (right) shows the elements in the whole 30S subunit. Figure made with RIBBONS⁵⁰.

particular, the binding to ribosomal RNA of S8 (refs 34, 35) and S15 (refs 36, 37) has been studied extensively.

After independent fitting of double-stranded RNA and proteins into electron density, a number of protein–RNA complexes were apparent, such as the adjacent S6–RNA and S15–RNA complexes (Fig. 2d). The extensive biochemical data on protein–RNA interactions in the central domain, the high density of proteins of previously known structure, and the well ordered density in this region allowed us to determine the fold of the entire central domain of 16S RNA. It also allowed us to examine details of its interaction with three proteins of known structure in this region, S6, S8 and S15. Furthermore, because of the known association of S6 and S18 in the absence of RNA and the proximity of S11 to S6, we could identify the location and low-resolution structure *in situ* of parts of these proteins (data not shown).

The central-domain RNA is an elongated, curved structure that wraps partly around the body of the small subunit (Fig. 3b, c). As seen elsewhere in the structure, the domain contains many short RNA helices packed together. A common mode of RNA–RNA packing involves the insertion of a ridge of phosphates into the minor groove of an adjacent double helix (for example, H26–H22 and H23a–H26 in Fig. 3c). Internal loops are used to modulate groove widths and to present purine N1 or N2 functional groups for helix–helix docking (for example, H20); they are also often found at sharp bends (such as the bends in H23 and H24).

The three-way junction of RNA helices 20, 21 and 22 is the heart of the central domain (Fig. 4). Its conformation is stabilized by the binding of S15 to the minor grooves of H20 and H22, in excellent agreement with *in vitro* biochemical studies on the interaction of S15 with fragments of 16S RNA^{36,37}. The tip of S15 is also close to H21 near the three-way junction, which is known to be required for S15 binding. The junction conformation is further stabilized by more tenuous interactions from S17 and S8, whose primary binding sites are on nearby helices. The S8–RNA interaction is in excellent agreement with binding studies that identify its interaction with a distorted minor groove in H21 (ref. 38) and a crosslink between Lys 55 of *E. coli* S8 and U653 (ref. 39), as well as footprinting studies²³ that show protection of both H21 identified in other studies and H25. In our structure, the amino-terminal helix of S8 makes contact with a minor groove of H25. Although S17 is very close to H21, and Lys 29 of *E. coli* S17 has been crosslinked to U632 (ref. 39), its interaction with this helix as seen in our map is slight, explaining a lack of footprint of S17 to this region. Rather, its RNA-binding regions interact with other double-helical RNA, presumably including H11 in the 5' domain (nucleotides 240–290).

The platform

The front half of the platform contains only the RNA helices H23 and H24, which terminate in the functionally important 690 and 790 loops. The 690 and 790 loops are indeed in close proximity (Fig. 3c), as has been predicted by on the basis of intra-site crosslinking⁴⁰ and footprinting experiments using P-site tRNA or antibiotics²⁸. Moreover, the 690 and 790 stems protrude into the intersubunit interface, with their minor grooves available for association with the 50S subunit, in agreement with hydroxyl-radical

footprinting data on subunit association³⁰.

H21 joins the platform to the back of the body, and H25, H19 and H27 are part of the body. Although H27, which is known to function in a switch, is well separated from the other helices, it makes a minor-groove packing interaction with H24. Moreover, the tip of H27 is close to ribosomal protein S5. Mutations in S5 have been implicated in the switch that is part of the role of H27 in accuracy³¹.

The platform appears to consist of two halves separated by a plane of lesser electron density, with this plane oriented roughly parallel to the intersubunit surface. Several observations indicate that the front and back halves of the platform may indeed exhibit some flexibility relative to each other. It is known that the platform is a flexible part of the subunit; electron-microscopy studies have shown that, upon subunit association or IF-3 binding, the platform moves upwards^{8,41}. Moreover, the separation between the two halves coincides roughly with the maximum extent of the proteins in this region, as if there is a limit to how close to the subunit interface protein-mediated stabilization is permissible. In addition, our fold of the central domain RNA reveals that this region of lesser density consists of potentially flexible multi-stem junctions. The maximal extent of hydroxyl-radical footprinting from 50S subunit association³⁰ extends approximately to the same vertical plane of lesser electron density. All of this evidence indicates that the platform has two halves which may be able to move independently relative to one another during translation.

The H27 accuracy switch

This putative motion of the front of the platform may be coupled to conformation changes in the decoding site by the H27 accuracy switch. The hairpin loop of H27 packs against the minor groove of the proximal end of H24 in the platform (Figs 3c, 5). If the front half of the platform does move independently, then H27 may well move with it. The other end of H27 makes a very extensive minor-groove packing interaction with H44 (Fig. 5), just below the decoding site where the A-site tRNA binds. This end of H27 is also close to ribosomal protein S5, and to RNA nucleotides footprinted by streptomycin; both streptomycin and mutations in S5 affect translational accuracy³¹. Previously, it was unknown how the H27 switch affected translational accuracy; now it appears likely that the H27 switch determines translation accuracy by modulating the conformation of the decoding site by a direct interaction, including a possible movement of H44.

Protein–protein interactions

Although many of the interactions of ribosomal proteins are with RNA, even within just the subset of proteins identified there is an example of interesting protein–protein interactions. Protein S5 forms close interactions with both S4 and S8. As shown in Fig. 6, the helical domain of S4 is juxtaposed against a face of S5, while the carboxy-terminal end of S5 makes an interface with S8. The mutations on S4 and S5 that confer the *ram* phenotype^{42,43} lie at their interface, indicating that in this case the mutations may disrupt a crucial protein–protein interaction rather than a protein–RNA contact, as previously suggested¹¹. Similarly, the interface between S5 and S8 is entirely consistent with the observed crosslink between S8 and the C-terminal region of S5 (ref. 44).

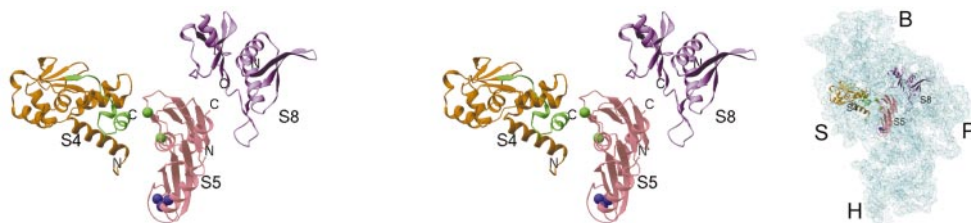


Figure 6 Stereo view of the relative disposition of proteins S4, S5 and S8 as they occur in the 30S subunit. The sites of *ram* mutations in S4 and S5 are shown in green (see text); the spectinomycin-resistance mutations in S5 are shown in blue.

Inset (right) shows the structures in the envelope of the 30S subunit. Figure made with RIBBONS⁵⁰.

We have compared portions of our model with the corresponding parts of a 30S model based on cryo-electron microscopy and biochemical data (F. Mueller, I. Sommer and R. Brimacombe, personal communication). The r.m.s.d. of the positions of proteins S4, S5, S8 and S15 in a least-squares superposition is 17.5 Å. In addition, the location of H21 in the model is similar to ours. However, the orientation of S5 relative to S4 is different, so that the *ram* sites on S5 do not appear to be part of the interface with S4. Both the orientation and location of S15 are significantly different. Finally, the quasi-continuous helices formed by stacking at three-way junctions in our structure of the central domain do not appear to be observed, so that while the locations of the other RNA helices in the central domain are close, as can be expected from topological constraints, they do not agree in detail.

Other regions of the 30S subunit

We have determined the locations of many double-helical RNA segments throughout the subunit. As ribosomal RNA double helices can be highly distorted because of bulges and non-canonical base pairs, this was done by visual inspection of the map rather than by automated search algorithms. These segments are shown in Fig. 7, along with the central domain, the proteins of known structure that have been identified in the map and our structure for S20. As shown in Fig. 7, our putative S20 is surrounded by RNA double helices, one of which is also close to S17. This result is consistent with data from

hydroxyl-radical footprinting²³ and nuclease protection²⁶. In this view, S20 would participate in the folding of the bottom portion of the 30S subunit and be intimately associated with 16S RNA. Its dual identification as the 50S protein L26 is therefore puzzling.

The bottom part of the 30S is relatively free of proteins. Although the head has several ribosomal proteins, none of their structures are known, with the exception of S7. We have therefore postponed a detailed interpretation of other regions of the 30S subunit until we have further analysed the current map and improved the resolution.

Discussion

One of the most striking features of the structure as a whole is the almost complete absence of protein from the subunit interface (Fig. 7b). Nearly all of the proteins identified in our map are located on either the sides or the back of the body. Only a small portion of S7 and possibly part of S12 appear to lie close to the subunit interface. This observation is consistent with predictions that the subunit interface contains little or no protein⁴⁵. As the interface is likely to be functionally the most important part of the ribosome, it also supports speculations that a primordial ribosome could have contained no protein at all.

However, our 30S structure also provides evidence that at least a few ribosomal proteins may be more directly involved in ribosomal function than previously suspected, as noted above for S4 and S5. Thus, although a primordial ribosome may have consisted entirely

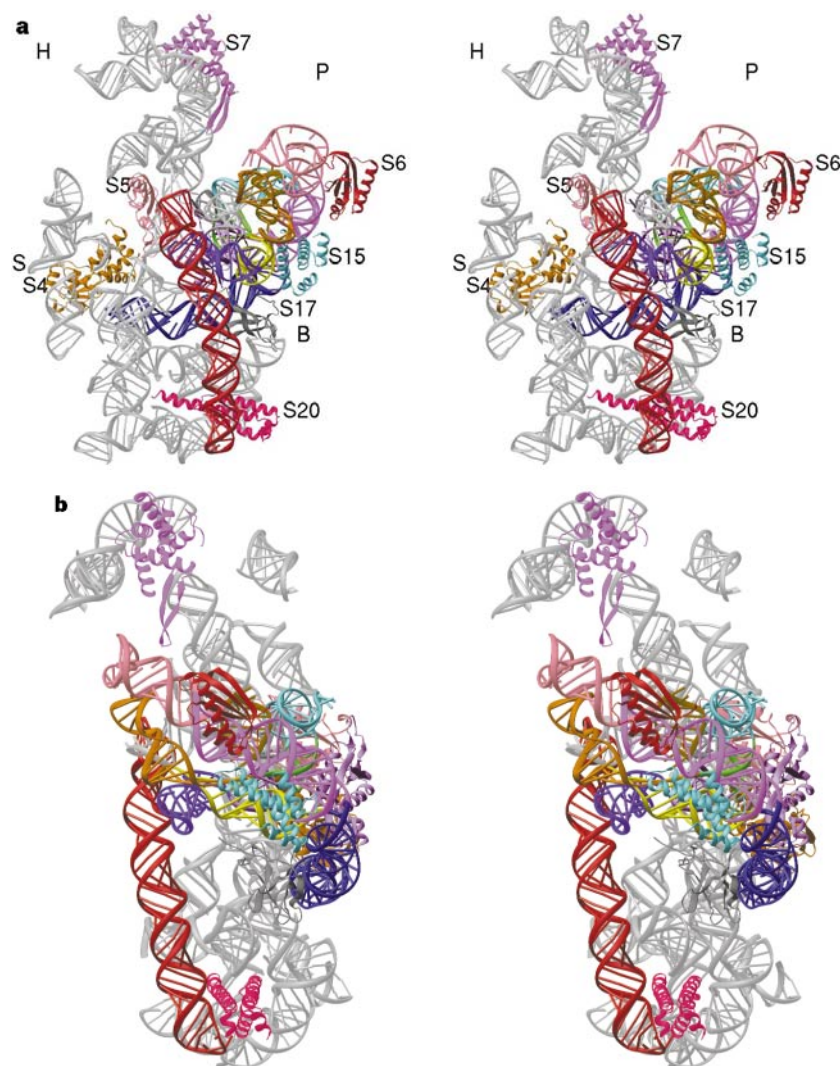


Figure 7 Two orthogonal stereo views of the 30S subunit. The central domain RNA is in the colouring scheme of Fig. 3, the 1,400/1,500 stem-loop of 16S RNA in red, the proteins of known structure that have been identified in the map, and

protein S20 near the bottom of the subunit. Other regions that have been identified as double-helical RNA are shown in grey. **a**, Intersubunit interface side faces the reader; in **b** it faces left. Figure made using RIBBONS⁵⁰.

of RNA, it is likely that not all of the moving parts and crucial surfaces in the ribosomes of today will be found to consist of RNA.

Our results have revealed the details of protein and RNA architecture in the 30S subunit, and the principles of their organization in its central domain. They also provide a firm basis for future biochemical and crystallographic analysis of the small subunit and its functions. □

Methods

Preparation and crystallization of ribosomal subunits. Ribosomes and 30S subunits were prepared from *T. Thermophilus* cells as described⁸. Crystals of 30S ribosomes were grown using the hanging-drop method with MPD as the precipitant, with minor modifications of the procedure of ref. 3. Crystals grew to a maximum size of $80 \times 80 \times 200 \mu\text{m}$, and were in space group $P4(1)2(1)2$ with cell dimensions of $a = b = 401.5 \text{ \AA}$ and $c = 174.5 \text{ \AA}$. The correct choice of space group between the two possible enantiomers was determined eventually by the handedness of RNA double helices in the electron-density map. Crystals were transferred to a stabilizing solution containing 26% MPD as a cryoprotectant (with or without heavy atom compounds) before flash-freezing in liquid nitrogen for data collection at 100 K.

Data collection. Data were collected on beamlines X12B and X25 at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory. We collected data on native and heavy atom derivatives of the crystals using $20 \times 20 \text{ cm}$ CCD detectors, the ADSC Quantum 4 detector on X12B or the Brandeis B4 detector on X25. The final data used for phasing were all from beamline X25. All data were indexed, scaled and reduced using the programs Denzo and Scalepack⁴⁶.

Phasing and analysis of maps. A Harker section of the difference Patterson map of data from a W17 cluster showed a clear strong peak of about 7 standard deviations above background, as well as a second minor peak. The positions of these sites and phases to low resolution were calculated using the program SOLVE⁴⁷. Difference Fouriers using these low-resolution phases revealed a substantial number of sites in the other heavy-atom derivatives. We used the program SOLVE both to determine independently and to confirm these sites, as well as to calculate phases by Bayesian phase refinement. The single native data set was not used in the phasing because of lack of isomorphism, and the osmium data set was used as the 'native' or reference data set in the phasing.

Density modification using the program SOLOMON⁴⁸ was used to improve the initial phases obtained from SOLVE. All map visualization and interpretation was done using the program O⁴⁹.

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- Alberts, B. *et al.* *Molecular Biology of the Cell* (Garland, New York, 1995).
- von Böhlen, K. *et al.* Characterization and preliminary attempts for derivatization of crystals of large ribosomal subunits from *Haloarcula marismortui* diffracting to 3 Å resolution. *J. Mol. Biol.* **222**, 11–15 (1991).
- Trakhanov, S. D. *et al.* Crystallization of 70 S ribosomes and 30 S ribosomal subunits from *Thermus thermophilus*. *FEBS Lett.* **220**, 319–322 (1987).
- Yusupov, M. M., Tischenko, S. V., Trakhanov, S. D., Ryazantsev, S. N. & Garber, M. B. A new crystalline form of 30 S ribosomal subunits from *Thermus thermophilus*. *FEBS Lett.* **238**, 113–115 (1988).
- Yonath, A. *et al.* Characterization of crystals of small ribosomal subunits. *J. Mol. Biol.* **203**, 831–834 (1988).
- Yonath, A. *et al.* Crystallographic studies on the ribosome, a large macromolecular assembly exhibiting severe nonisomorphism, extreme beam sensitivity and no internal symmetry. *Acta Crystallogr. A* **54**, 945–955 (1998).
- Lata, K. R. *et al.* Three-dimensional reconstruction of the *Escherichia coli* 30S ribosomal subunit in ice. *J. Mol. Biol.* **262**, 43–52 (1996).
- McCutcheon, J. P. *et al.* Location of translational initiation factor IF3 on the small ribosomal subunit. *Proc. Natl Acad. Sci. USA* **96**, 4301–4306 (1999).
- Mueller, F. & Brimacombe, R. A new model for the three-dimensional folding of *Escherichia coli* 16 S ribosomal RNA. I. Fitting the RNA to a 3D electron microscopic map at 20 Å. *J. Mol. Biol.* **271**, 524–544 (1997).
- Allain, F. H. & Varani, G. Structure of the P1 helix from group I self-splicing introns. *J. Mol. Biol.* **250**, 333–353 (1995).
- Ramakrishnan, V. & White, S. W. Structure of ribosomal protein S5 reveals sites of interaction with 16S RNA. *Nature* **358**, 768–771 (1992).
- Lindahl, M. *et al.* Crystal structure of the ribosomal protein S6 from *Thermus thermophilus*. *EMBO J.* **13**, 1249–1254 (1994).
- Jaishree, T. N., Ramakrishnan, V. & White, S. W. Solution structure of prokaryotic ribosomal protein S17 by high-resolution NMR spectroscopy. *Biochemistry* **35**, 2845–2853 (1996).
- Davies, C., Ramakrishnan, V. & White, S. W. Structural evidence for specific S8–RNA and S8–protein interactions within the 30S ribosomal subunit; ribosomal protein S8 from *Bacillus stearothermophilus* at 1.9 Å resolution. *Structure* **4**, 1093–1104 (1996).
- Berglund, H., Rak, A., Serganov, A., Garber, M. & Härd, T. Solution structure of the ribosomal RNA binding protein S15 from *Thermus thermophilus*. *Nature Struct. Biol.* **4**, 20–23 (1997).
- Winberly, B. T., White, S. W. & Ramakrishnan, V. The structure of ribosomal protein S7 at 1.9 Å resolution reveals a β-hairpin motif that binds double-stranded nucleic acids. *Structure* **5**, 1187–1198 (1997).

- Hosaka, H. *et al.* Ribosomal protein S7: a new RNA-binding motif with structural similarities to a DNA architectural factor. *Structure* **5**, 1199–1208 (1997).
- Clemons, W. M. Jr, Davies, C., White, S. W. & Ramakrishnan, V. Conformational variability of the N-terminal helix in the structure of ribosomal protein S15. *Structure* **6**, 429–438 (1998).
- Nevskaya, N. *et al.* Crystal structure of ribosomal protein S8 from *Thermus thermophilus* reveals a high degree of structural conservation of a specific RNA binding site. *J. Mol. Biol.* **279**, 233–244 (1998).
- Davies, C., Gerstner, R. B., Draper, D. E., Ramakrishnan, V. & White, S. W. The crystal structure of ribosomal protein S4 reveals a two-domain molecule with an extensive RNA-binding surface: one domain shows structural homology to the ETS DNA-binding motif. *EMBO J.* **17**, 4545–4558 (1998).
- Markus, M. A., Gerstner, R. B., Draper, D. E. & Torchia, D. A. The solution structure of ribosomal protein S4 delta41 reveals two subdomains and a positively charged surface that may interact with RNA. *EMBO J.* **17**, 4559–4571 (1998).
- Capel, M. S. *et al.* A complete mapping of the proteins in the small ribosomal subunit of *Escherichia coli*. *Science* **238**, 1403–1406 (1987).
- Powers, T. & Noller, H. F. Hydroxyl radical footprinting of ribosomal proteins on 16S rRNA. *RNA* **1**, 194–209 (1995).
- Mueller, F. & Brimacombe, R. A new model for the three-dimensional folding of *Escherichia coli* 16 S ribosomal RNA. II. The RNA–protein interaction data. *J. Mol. Biol.* **271**, 545–565 (1997).
- Ramakrishnan, V. *et al.* Position of proteins S6, S11 and S15 in the 30 S ribosomal subunit of *Escherichia coli*. *J. Mol. Biol.* **153**, 739–760 (1981).
- Ungewickell, E., Garrett, R., Ehresmann, C., Stiegler, P. & Fellner, P. An investigation of the 16-S RNA binding sites of ribosomal proteins S4, S8, S15, and S20 from *Escherichia coli*. *Eur. J. Biochem.* **51**, 165–180 (1975).
- Mueller, F., Stark, H., van Heel, M., Rinke-Appel, J. & Brimacombe, R. A new model for the three-dimensional folding of *Escherichia coli* 16 S ribosomal RNA. III. The topography of the functional centre. *J. Mol. Biol.* **271**, 566–587 (1997).
- Moazed, D. & Noller, H. F. Binding of tRNA to the ribosomal A and P sites protects two distinct sets of nucleotides in 16 S rRNA. *J. Mol. Biol.* **211**, 135–145 (1990).
- Lee, K., Varma, S., SantaLucia, J. Jr & Cunningham, P. R. *In vivo* determination of RNA structure–function relationships: analysis of the 790 loop in ribosomal RNA. *J. Mol. Biol.* **269**, 732–743 (1997).
- Merryman, C., Moazed, D., McWhirter, J. & Noller, H. F. Nucleotides in 16S rRNA protected by the association of 30S and 50S ribosomal subunits. *J. Mol. Biol.* **285**, 97–105 (1999).
- Lodmell, J. S. & Dahlberg, A. E. A conformational switch in *Escherichia coli* 16S ribosomal RNA during decoding of messenger RNA. *Science* **277**, 1262–1267 (1997).
- Gregory, R. J. *et al.* Interaction of ribosomal proteins S6, S8, S15 and S18 with the central domain of 16 S ribosomal RNA from *Escherichia coli*. *J. Mol. Biol.* **178**, 287–302 (1984).
- Greuer, B., Osswald, M., Brimacombe, R. & Stöffler, G. RNA-protein cross-linking in *Escherichia coli* 30S ribosomal subunits; determination of sites on 16S RNA that are cross-linked to proteins S3, S4, S7, S9, S10, S11, S17, S18 and S21 by treatment with bis-(2-chloroethyl)-methylamine. *Nucleic Acids Res.* **15**, 3241–3255 (1987).
- Wu, H., Jiang, L. & Zimmermann, R. A. The binding site for ribosomal protein S8 in 16S rRNA and spc mRNA from *Escherichia coli*: minimum structural requirements and the effects of single bulged bases on S8–RNA interaction. *Nucleic Acids Res.* **22**, 1687–1695 (1994).
- Moine, H., Cachia, C., Westhof, E., Ehresmann, B. & Ehresmann, C. The RNA binding site of S8 ribosomal protein of *Escherichia coli*: Slex and hydroxyl radical probing studies. *RNA* **3**, 255–268 (1997).
- Batey, R. & Williamson, J. Interaction of the *Bacillus stearothermophilus* ribosomal protein S15 with 16 S rRNA: I. Defining the minimal RNA site. *J. Mol. Biol.* **261**, 536–549 (1996).
- Serganov, A. A. *et al.* The 16S rRNA binding site of *Thermus thermophilus* ribosomal protein S15: comparison with *Escherichia coli* S15, minimum site and structure. *RNA* **2**, 1124–1138 (1996).
- Kalurachi, K., Uma, K., Zimmermann, R. A. & Nikonowicz, E. P. Structural features of the binding site for ribosomal protein S8 in *Escherichia coli* 16S rRNA defined using NMR spectroscopy. *Proc. Natl Acad. Sci. USA* **94**, 2139–2144 (1997).
- Urlaub, H., Thiede, B., Müller, E. C., Brimacombe, R. & Wittmann-Liebold, B. Identification and sequence analysis of contact sites between ribosomal proteins and rRNA in *Escherichia coli* 30 S subunits by a new approach using matrix-assisted laser desorption/ionization-mass spectrometry combined with N-terminal microsequencing. *J. Biol. Chem.* **272**, 14547–14555 (1997).
- Atmadja, J. *et al.* The tertiary folding of *Escherichia coli* 16S RNA, as studied by *in situ* intra-RNA cross-linking of 30S ribosomal subunits with bis-(2-chloroethyl)-methylamine. *Nucleic Acids Res.* **14**, 659–673 (1986).
- Agrawal, R. K., Penczek, P., Grassucci, R. A. & Frank, J. Visualization of elongation factor G on the *Escherichia coli* 70S ribosome: the mechanism of translocation. *Proc. Natl Acad. Sci. USA* **95**, 6134–6138 (1998).
- van Acken, U. Proteinchemical studies on ribosomal proteins S4 and S12 from ram (ribosomal ambiguity) mutants of *Escherichia coli*. *Mol. Gen. Genet.* **140**, 61–68 (1975).
- Wittmann-Liebold, B. & Greuer, B. The primary structure of protein S5 from the small subunit of the *Escherichia coli* ribosome. *FEBS Lett.* **95**, 91–98 (1978).
- Allen, G., Capasso, R. & Gualerzi, C. Identification of the amino acid residues of proteins S5 and S8 adjacent to each other in the 30 S ribosomal subunit of *Escherichia coli*. *J. Biol. Chem.* **254**, 9800–9806 (1979).
- Agafonov, D. E., Kolb, V. A. & Spirin, A. S. Proteins on ribosome surface: measurements of protein exposure by hot tritium bombardment technique. *Proc. Natl Acad. Sci. USA* **94**, 12892–12897 (1997).
- Otwiński, Z. & Minor, W. in *Methods in Enzymology* (eds Carter, C. W. J. & Sweet, R. M.) 307–325 (Academic, New York, 1997).
- Terwilliger, T. & Berendzen, J. Automated MAD and MIR structure determination. *Acta Crystallogr. D* **55**, 849–861 (1999).
- Abrahams, J. P. & Leslie, A. G. W. Methods used in the structure determination of bovine mitochondrial F1 ATPase. *Acta Crystallogr. D* **52**, 30–42 (1996).
- Jones, T. A. & Kjeldgaard, M. Electron-density map interpretation. *Methods Enzymol.* **277B**, 173–207 (1997).
- Carson, M. Ribbons 2.0. *J. Appl. Crystallogr.* **24**, 958–961 (1991).

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Placement of protein and RNA structures into a 5 Å-resolution map of the 50S ribosomal subunit

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We have calculated at 5.0 Å resolution an electron-density map of the large 50S ribosomal subunit from the bacterium *Haloarcula marismortui* by using phases derived from four heavy-atom derivatives, intercrystal density averaging and density-modification procedures. More than 300 base pairs of A-form RNA duplex have been fitted into this map, as have regions of non-A-form duplex, single-stranded segments and tetraloops. The long rods of RNA crisscrossing the subunit arise from the stacking of short, separate double helices, not all of which are A-form, and in many places proteins crosslink two or more of these rods. The polypeptide exit channel was marked by tungsten cluster compounds bound in one heavy-atom-derivatized crystal. We have determined the structure of the translation-factor-binding centre by fitting the crystal structures of the ribosomal proteins L6, L11 and L14, the sarcin-ricin loop RNA, and the RNA sequence that binds L11 into the electron density. We can position either elongation factor G or elongation factor Tu complexed with an aminoacylated transfer RNA and GTP onto the factor-binding centre in a manner that is consistent with results from biochemical and electron microscopy studies.

The large (50S) ribosomal subunit catalyses the peptidyl-transfer reaction of messenger-RNA-directed protein synthesis¹. In bacteria, it consists of a ~2,900-nucleotide 23S RNA, a 120-nucleotide 5S RNA and about 33 different proteins; it has a relative molecular

mass of $\sim 1.6 \times 10^6$ (ref. 2). Peptide-bond synthesis occurs at the bottom of a deep cleft in the large ribosomal subunit where the aminoacylated acceptor stem of the tRNA carrying the next amino acid is brought together with the corresponding region of a

Table 1 Statistics for data collection and phase determination

Space group:	C222 ₁						P2 ₁	C222 ₁
Unit-cell dimensions (Å):	212 × 301 × 576						184 × 570 × 184 β = 108.5	210 × 300 × 540
Asymmetric unit:	1 molecule						2 molecules	1 molecule
	Native 1	Native 2	W ₁₈	W ₁₁	TA	OsHx	HM50SP21	HM50SD
Soaking time (days)	–	–	6	2	5	2	–	–
Wavelength (Å)	1.21	1.34	1.21	1.21	1.25	1.14	1.14	1.21
Resolution (Å)	120–3.75	70–4.3	100–4.0	120–4.0	120–4.8	30–3.4	30–4.6	30–3.6
Observations	957,850	496,354	1,467,476	1,411,640	366,832	1,646,468	1,144,780	600,063
Unique	185,190	116,447	302,089	303,285	133,044	488,275	198,796	163,558
Completeness	98.6	93.6	99.9	99.6	74.3	97.9	100.0	83.2
R _{merge}	10.3	21.8	13.4	14.3	10.4	14.2	21.1	17.0
R _{merge} (ano)	–	–	8.7	9.1	6.9	7.4	–	–
Cluster conc. (mM)	–	–	1.0	1.0	1.0	4.5	–	–
No. of sites #	–	–	6	10	9	38	–	–
Riso (30–5.0 Å)	–	–	26.5	22.5	19.5	14.5	–	–
I/σ (highest res. bin)	13.0 (2.2)	6.2 (1.8)	10.4 (1.8)	8.9 (1.6)	10.6 (2.37)	9.9 (2.08)	8.6 (3.2)	7.6 (1.6)
MIRAS phasing statistics	Resolution shells (Å) – ~34,600 reflections per bin							
	60.0	6.9	5.8	5.2	5.0	Total		
W ₁₈								
Phasing power	1.05	0.32	0.46	0.54		1.00		
R _{cullis} (centric)	0.60	0.73	0.71	0.70		0.62		
W ₁₁								
Phasing power	1.09	0.35	0.41	0.43		1.00		
R _{cullis} (centric)	0.60	0.74	0.72	0.73		0.61		
TA								
Phasing power	0.92	0.30	0.10	0.10		0.81		
R _{cullis} (centric)	0.69	0.83	0.90	0.97		0.72		
OsHx								
Phasing power	0.52	0.40	0.33	0.25		0.40		
R _{cullis} (centric)	0.65	0.68	0.74	0.73		0.69		
(MIRAS/SAD) FOM	0.65	0.31	0.27	0.22		0.37		
(Density modification) FOM	0.94	0.62	0.32	0.18		0.51		

$R_{iso} = \Sigma |F_{PH} - F_P| / \Sigma F_{PH}$, where F_{PH} and F_P are the derivative and the native structure factor amplitudes, respectively; $R_{sym} = \Sigma |I(h) - I(h)| / \Sigma I(h)$, where $I(h)$ is the mean intensity of reflections. Phasing power: r.m.s. isomorphous difference divided by the r.m.s. residual lack of closure. $R_{cullis} = \Sigma (|F_{PH} - F_P| - |F_{H(calc)}|) / \Sigma |F_{PH} - F_P|$. The summation is valid only for centric reflections. W₁₈, W₁₁ and TA are the eighteen and eleven tungsten and six tantalum cluster compounds described earlier²¹ and OsHx is osmium hexamine. HM50SP21 is a monoclinic data set with a twinning fraction of 0.27. HM50SD is an orthorhombic data set with a shorter c-axis.

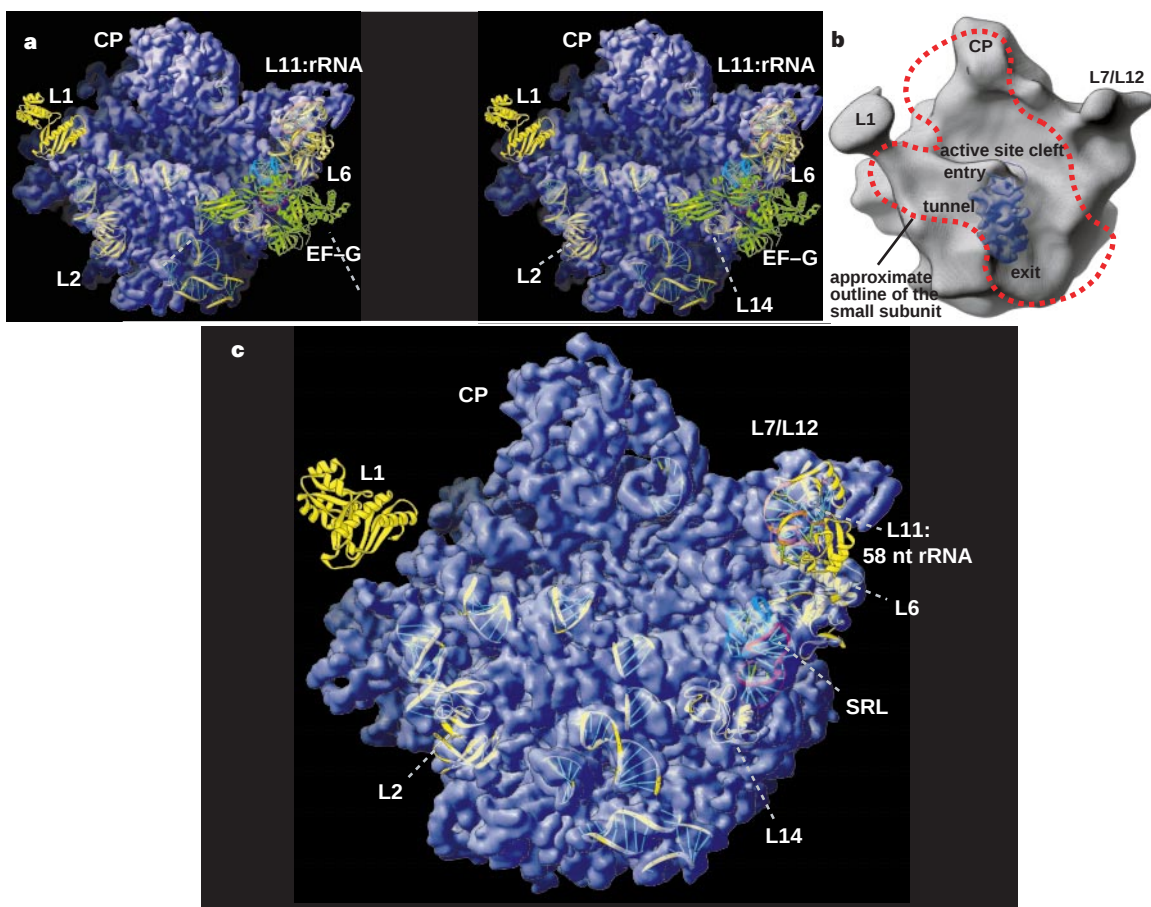


Figure 1 The 5Å-resolution electron-density map of the 50S ribosomal subunit. In **a** and **c**, some RNA helices and tetraloops of unknown assignment (in yellow) have been fitted into density to guide the viewer. The backbone of the SRL is red, the backbones of helices believed to be 96 and 97 are blue and yellow, respectively, and the backbone of the L11 RNA is yellow. All proteins are shown as ribbon structures. The structure of protein L1 (ref. 45) was positioned in a 12Å-resolution MIR map. **a**, A stereo representation of the subunit is viewed down the peptide synthesis active-site cleft. EF-G, in green, has been docked onto the fitted

proteins and RNA. **b**, Solid-surface drawing of the large subunit in the crown view and in the same orientation as in **c**. The locations of proteins L1, L7/L12 and the central protuberance, as determined by electron microscopy, are labelled. The tunnel seen at 5 Å resolution, is shown in blue and is viewed at a 45° angle to its long axis. **c**, 'Crown' view of the 50S subunit, including ribbon representations of protein and RNA-fragment structures that have been fitted to the density on this side of the subunit. Identified structures are labelled.

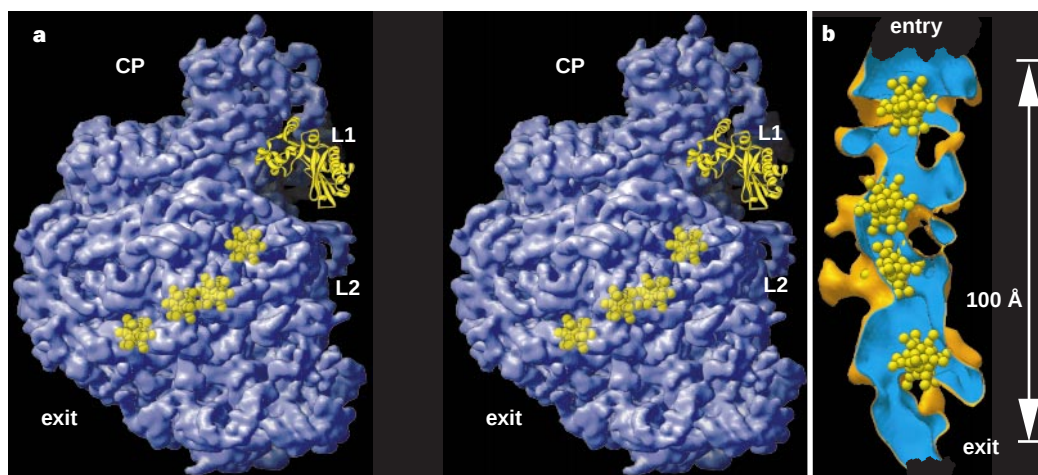


Figure 2 Visualization of the tunnel in which tungsten clusters are located. **a**, Stereo view, rotated by 90° about the vertical axis from the crown view (Fig. 1b, c) and showing the four tungsten clusters that mark the polypeptide exit tunnel. **b**,

Close-up for the tunnel sliced on one side to show the four tungsten clusters. The surface is gold on the outside and blue on the inside and represents the low electron density (empty space) in the proximity of the clusters.

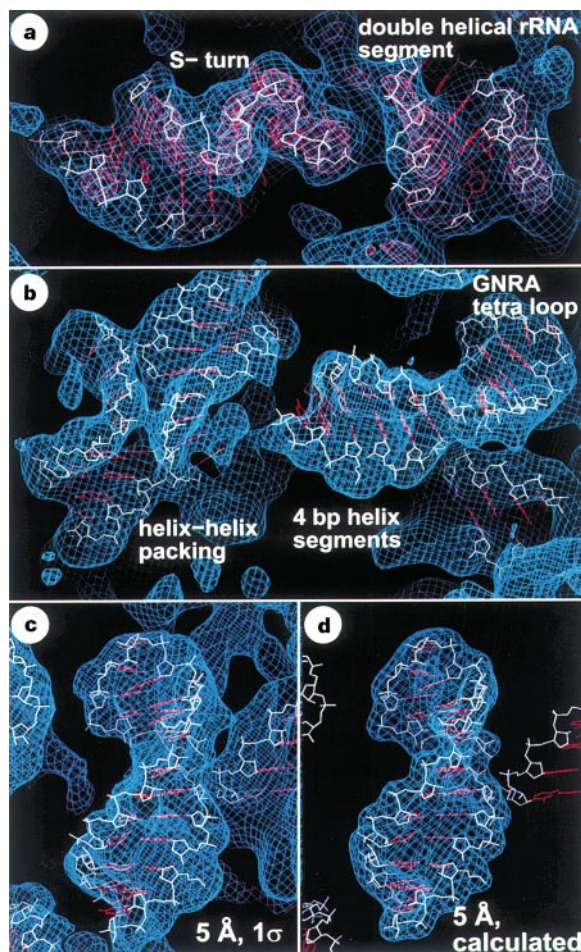


Figure 3 A variety of interactions between RNA duplexes. **a**, An RNA of unknown sequence and containing an S-turn backbone structure with its tetraloop is seen to be docked into an RNA duplex. **b**, Many RNA duplexes show direct interactions, some of which may involve ribose-ribose contacts. **c**, **d**, The experimental electron density map (**c**) and one calculated at 5 Å resolution from the atomic coordinates with a B-factor of 70 (**d**) are very similar. Bumps in the electron density arising from the phosphoribose backbone are seen in both.

peptidyl-tRNA^{3,4}. The elongating polypeptide is believed to exit through a tunnel that passes through the middle of the 50S subunit^{5,6}. This subunit also contains a region termed the 'GTPase-associated site' or the 'GTPase centre', which we call the 'factor-binding centre': this region includes proteins L6, L11, L14 and the L7/L12 stalk, as well as the sarcin-ricin loop sequence and the 1,055–1,080-nucleotide region of 23S RNA⁷. The factor-binding centre binds the two translation-elongation factors EF-G and EF-Tu, as well as the initiation factor IF-2 and the release factor RF-3, all of which are G proteins: binding to the centre stimulates their GTPase activity.

All of the proteins in the small ribosomal subunit⁸ and some in the large subunit⁹ have been positioned by neutron scattering, and immune electron microscopy has provided more approximate localizations of proteins in both subunits^{10,11}. X-ray crystallographic and NMR studies have determined the structures at high resolution of ten proteins of the large subunit¹², two RNA fragments (the sarcin-ricin loop (SRL)^{13,14} and half of 5S RNA^{15,16}), complexes of protein L11 with a 58-nucleotide RNA fragment^{17,18} and L25 complexed with a fragment of 5S RNA (M. Lu and T.A.S., unpublished results). Single-particle analysis of whole 70S ribosomes by cryo-electron microscopy has generated three-dimensional maps of the ribosome and its complexes with tRNA and factors, at resolu-

tions varying between 13 and 24 Å (refs 4, 19, 20). A 9 Å-resolution electron-density map of the 50S subunit obtained by X-ray crystallography revealed density consistent with long RNA rods that crisscross the subunit²¹.

We have now calculated at 5 Å resolution an X-ray crystallographic electron-density map of the 50S subunit which has enabled us to make atomic-level structural interpretations of this large assembly. Familiar generic structures, such as A-form RNA duplex and RNA tetraloops, as well as the crystal structures of specific RNA fragments and proteins, can be positioned in this map. We can now plausibly position the major RNA and protein components of the factor-binding centre in the subunit's structure and propose a model for the way in which elongation factors bind to it.

Structure determination

The 5 Å-resolution map was obtained using multiple isomorphous replacement (MIR)/single wavelength anomalous difference (SAD) phasing together with intercrystal and non-crystallographic averaging between three different crystal forms (Table 1). This was possible only after we discovered that the *H. marismortui* 50S subunit crystallizes both in an orthorhombic space group, $C222_1$, having unit-cell dimensions $a = 212$, $b = 301$ and $c = 576$ Å, and in a merohedrally twinned monoclinic space group, $P2_1$, whose cell dimensions and Laue symmetry are nearly indistinguishable from those of the orthorhombic form but whose diffraction intensities differ by about 30–35% (30 to 5.5 Å resolution data). The space-group change and the twinning were discovered by examination of difference Patterson maps of the two crystal forms derivatized with a tungsten cluster (W_{18}) compound. Twinning could not be detected by analysing intensity statistics, perhaps because of strong pseudo- $C222_1$ symmetry in each of the monoclinic crystals, or because only low-resolution data were available. Using a 20 Å-resolution reconstruction of the *H. marismortui* large ribosomal subunit²¹, we found a molecular replacement solution for the two subunits in the asymmetric unit of the $P2_1$ crystals, which explains the W_{18} difference-Patterson map in $P2_1$ and confirms that the $P2_1$ crystals are twinned. The subunit packing in the $P2_1$ and $C222_1$ crystals differs by a 10 Å translation, which eliminates two dyad axes of the orthorhombic crystals (see Methods).

All phasing by heavy-atom methods was performed in the orthorhombic unit cell. An osmium hexamine derivative was found, and by combining data from it with the data from the original three heavy-atom derivatives, MIRAS phases could be obtained that had a satisfactory figure of merit to 7 Å and contained significant phase information beyond that. Phases were improved beyond 7 Å by combining SAD phases obtained from each derivative with four-derivative MIRAS phases and by intercrystal averaging^{22,23} using two non-isomorphous orthorhombic data sets and two detwinned monoclinic data sets (Table 1).

General structure at 5 Å resolution

The particle envelope of the 5 Å-resolution map has the same overall shape (Fig. 1) as that obtained previously by cryo-electron microscopy at 20 Å resolution²¹. At the higher resolution, however, deep crevices and open spaces are seen to separate large domains. One of the most prominent features of the subunit is a very deep, wide cleft that forms the entrance to the peptidyltransferase active site. The 'rim' of this cleft consists largely of a long rod of RNA duplex constructed from a series of stacked double-stranded helices that apparently emanate from several different regions of the 23S RNA sequence.

At the bottom of the active-site cleft is a tunnel that runs straight through the subunit. In one of our heavy-atom derivatized crystals, the path of this tunnel is marked by the positions of four bound eleven-tungsten(W_{11})-cluster molecules, which have a van der Waals diameter of nearly 20 Å and lie on an almost straight line (Fig. 2). This tunnel is thought to be the path used by the nascent

polypeptide chain to traverse from the peptidyltransferase centre to the point where it leaves the ribosome^{6,24}, an idea supported by the location at the tunnel entrance of the aminoacyl end of formyl-methionine (fMet)-tRNA bound at the P-site⁴ and the alignment of the tunnel exit on the back of the large subunit with the central pore of the Sec61 channel in yeast⁶.

The 5Å-resolution electron-density map of the 50S ribosomal subunit shows well resolved density for the backbone of the two strands of duplex RNA and higher density features at the positions of phosphate groups. Density for base pairs is continuous and smoothly joins the backbone of the two strands. The shape of the density corresponding to RNA seen in the experimental 5Å-resolution map is very similar to that observed in a perfect 5Å-resolution map produced using calculated phases (Fig. 3c), indicating that the experimental map is reasonably well phased to 5 Å resolution. Using the real-space correlation program ESSENS²⁵ and visual inspection, density corresponding to more than 300 base pairs of A-form duplex RNA has been fitted to models.

Long rods of double-stranded RNA crisscross the subunit, and helices of A-form and non-A-form duplex RNA from different sequence regions stack to form these rods (Fig. 3). There are also regions where RNA helices lying side by side interact (Fig. 3), reminiscent of arrangements seen in the structure of the P4-6 domain of the group I ribozyme²⁶, and some of these inter-RNA duplex interactions may involve contacts between backbone ribose groups (ribose 'zippers'). Another well known structural motif seen repeatedly in these maps is an RNA tetraloop of sequence guanine-any nucleotide-purine-adenine (GNRA)²⁷, which is often found at the ends of duplex hairpins (Figs 3, 4). Although many regions of single-stranded RNA can be seen in these 5Å-resolution maps, they are difficult to trace unambiguously for long distances. Density arising from the protein can be differentiated from that attributed to RNA because of its different character. The rods of density arising from α -helices are the easiest to identify and the hardest are the β -sheet regions.

So far about 12 proteins have been located (although no systematic search has been undertaken) and the atomic coordinates of five proteins have been fitted to the density. Most of these proteins are evenly dispersed among RNA regions, consistent with IEM localization^{10,11} and crosslink two or more RNA rods. They neither cover the RNA, as occurs in viruses, nor are they surrounded by nucleic acid, as in the nucleosome. Many examples of protein α -helices interacting with the minor or major grooves of duplex RNA have been found, as well as some β -strand interactions. Most of the proteins seem to fix the tertiary structure of 23S rRNA by cross-linking helical segments that are distant in the sequence, which indicates that they may be important in stabilizing ribosome conformation, consistent with biochemical observations²⁸.

The structure of the factor-binding centre

We have been able to position the crystal structures of three proteins and two RNA fragments that form a major part of the binding centre for G-protein factors. Using the program ESSENS, we first found the electron density corresponding to the sarcin-ricin loop (SRL), which then guided us to L6 (Fig. 4). The SRL was identifiable because of its unusual S-shaped backbone structure, which is close to a GNRA tetraloop. Only one other such electron-density region was found in the map (Fig. 3), but its location is inconsistent with other data on the position of the SRL⁹. Protein L6 was identified primarily by its two long α -helices, which are in different domains²⁹. To position these helices in the long rods of density to which they correspond, the angle between the domains had to be altered by about 5°. The β -sheet regions then fitted well into regions of broad density (Fig. 4), an interpretation that would not have been possible in the absence of a high-resolution crystal structure.

Biochemical and genetic data on the interacting regions of the SRL and L6 are in agreement with the structure derived from the 5Å-resolution map. Dimethyl sulphate protection and crosslinking data show that L6 binds to domain VI of 23S rRNA, particularly to

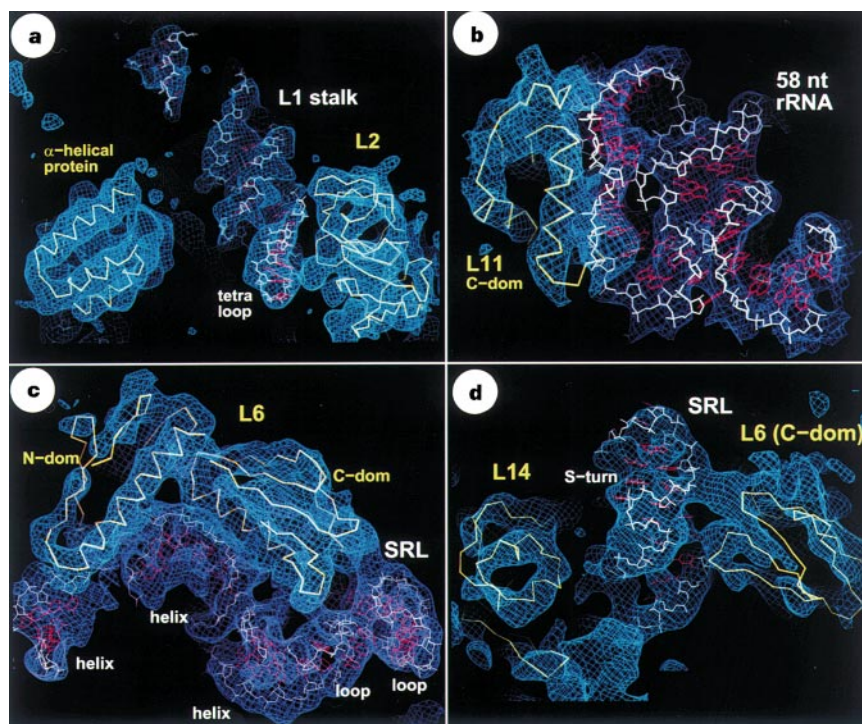


Figure 4 The fitting of previously solved protein and nucleic acid structures to the 5Å-resolution electron density map. **a**, The known crystal structure of a protein L2 fragment³⁶ and an unknown α -helical protein are seen to be interacting with an RNA helix that forms the base of a stalk on which protein L1 (not shown) sits. **b**, Crystal structure of the protein L11 C-terminal domain complexed with 58

nucleotides of rRNA^{20,21} fitted into the electron density. **c**, The crystal structures of protein L6 (ref. 29) and the sarcin-ricin loop^{16,17}, as well as two other RNA regions are fitted into density. **d**, The density identified as the SRL shows the characteristic S-turn in the RNA backbone. Proteins L14 (ref. 34) and L6 are seen to interact with the SRL on opposite sides.

helices 95 (SRL) and 97 (refs 30–32). The hydroxyl group of Tyr 156, which has been crosslinked to 23S rRNA³³, is less than 4 Å away from the backbone of a tetraloop that interacts with the SRL.

The structure of protein L14 (ref. 34) was subsequently found (Fig. 4c) by making use, in part, of the relative locations of L6 and L14 in the IEM map of the 50S subunit³⁵. Two crystal structures, one of L11 complexed with 58 nucleotides of RNA¹⁷ and the other of a single L11 domain bound to a similar RNA¹⁸, were then positioned (Fig. 4b) in the density, guided by the fact that this complex must be near the SRL in the L7/L12 stalk⁷. There is no electron density in our map for the N-terminal domain of L11, suggesting that this domain is not ordered in these crystals. Likewise, there is no density for the L7/L12 stalk, which is also expected to be intimately involved in factor binding^{7,20}. Nevertheless, the three located proteins and two RNA fragments constitute most of the factor-binding centre. The structure of an L2 protein fragment³⁶ was also positioned (Fig. 4a) using IEM data as a guide³⁵.

Docking aa-tRNA·EF-Tu·GTP and EF-G to 50S

Two types of data were used to position the elongation factors on the factor-binding centre of the 50S subunit. First, cryo-electron microscopy reconstructions of ribosomes complexed with either aa-

tRNA·EF-Tu·GTP or EF-G provide the approximate orientation of these two G proteins; both bind in a similar way at the base of the L7/L12 stalk^{19,20}. Second, a site-directed RNA hydrolysis has been used to provide distance constraints between specific residues on EF-G that have been mutated to cysteines and specific nucleotides in the 23S rRNA that have been hydrolysed by a reagent that can generate hydroxyl radicals, BABE, attached to the cysteine residues³⁷. The mutated and derivatized EF-G was bound in the post-translocation state by addition of GTP and fusidic acid. By positioning the two residues of EF-G as closely as possible to the RNA residues that were hydrolysed (Fig. 5) and simultaneously maximizing the steric complementarity between EF-G^{38,39} and the 50S subunit, EF-G can be docked in a position that is broadly consistent with the structure of this complex obtained by cryo-electron microscopy¹⁹. The aa-tRNA·EF-Tu·GTP (ref. 40) was then positioned by superimposing the corresponding G-domain α -carbon atoms, assuming that the homologous G domains of these two enzymes bind in an identical way (Fig. 5). Even though the shape complementarity between both factors and the electron density of the 50S subunit is good, the accuracy of this factor-docking model and its degree of correspondence with the cryo-electron microscopic density is difficult to assess because we do not

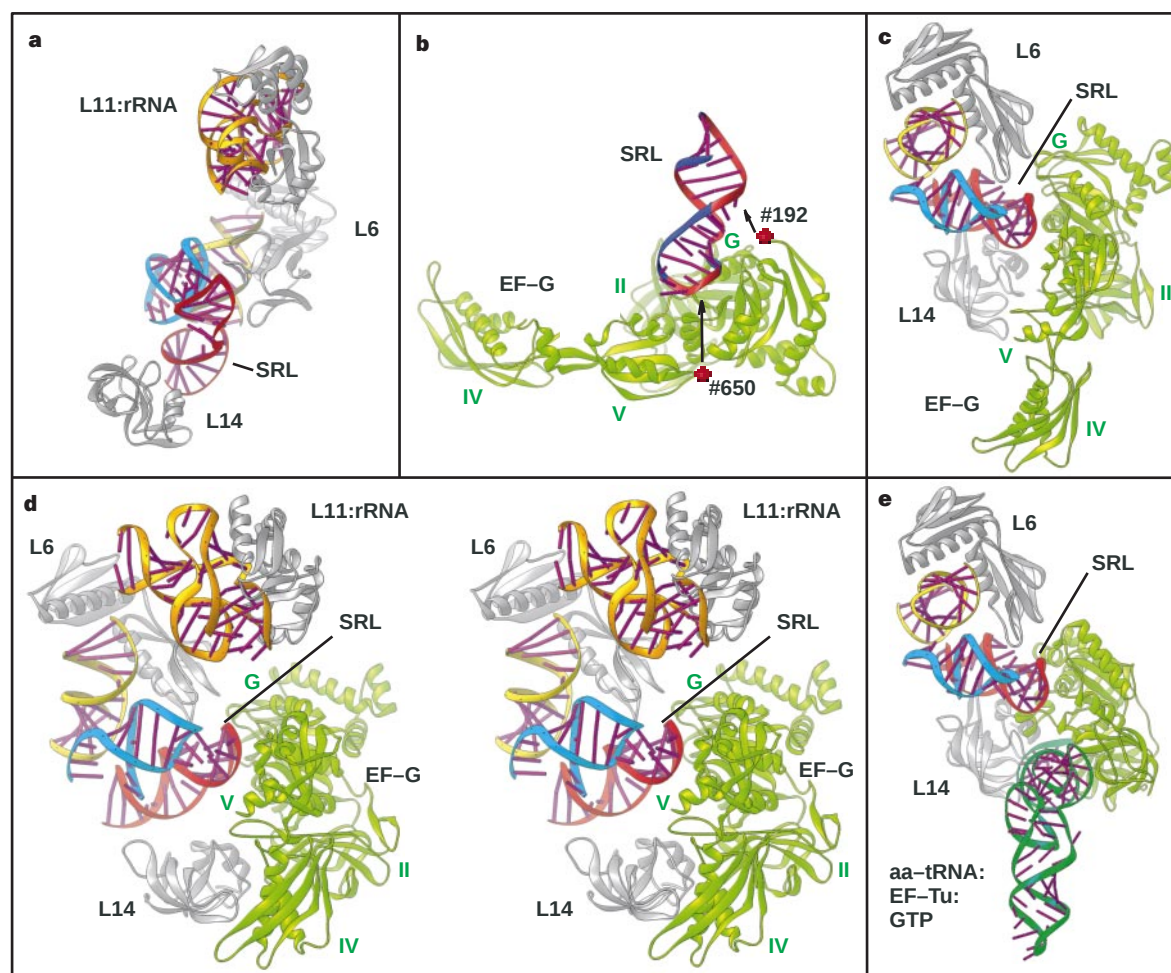


Figure 5 The proteins (in white backbone ribbons) and RNAs (in coloured ribbon) of the factor-binding site that have been fitted to the density. The docked structures of EF-G·GDP and aa-tRNA·EF-Tu·GTP are shown in green. **a**, The three proteins and three RNA fragments as fitted to the density are shown in the crown view (Fig. 1). **b**, Docking of EF-G on the SRL using site-directed RNA cleavage data³⁷. The backbone of EF-G, in blue, with residues 650 and 192, to which the BABE cleavage reagent was attached, is represented as red spheres. The SRL backbone cleaved by BABE bound to residues 650 and 192 is in red. The EF-G

domains are labelled in green^{39,40}. **c**, The factor-binding centre and the docked EF-G shown in a 'top' view (without L11:rRNA) demonstrate the shape complementarity between the centre and the EF-G. **d**, Another orientation of **c**, shown in stereo, that includes L11:rRNA, also shows the complementarity and proximity of structures. **e**, The centre proteins and rRNAs, shown and oriented as in **c**, but with aa-tRNA·EF-Tu·GTP (ref. 40) replacing EF-G, shows the proximity of the SRL (red) to the G domain and of L14 to tRNA.

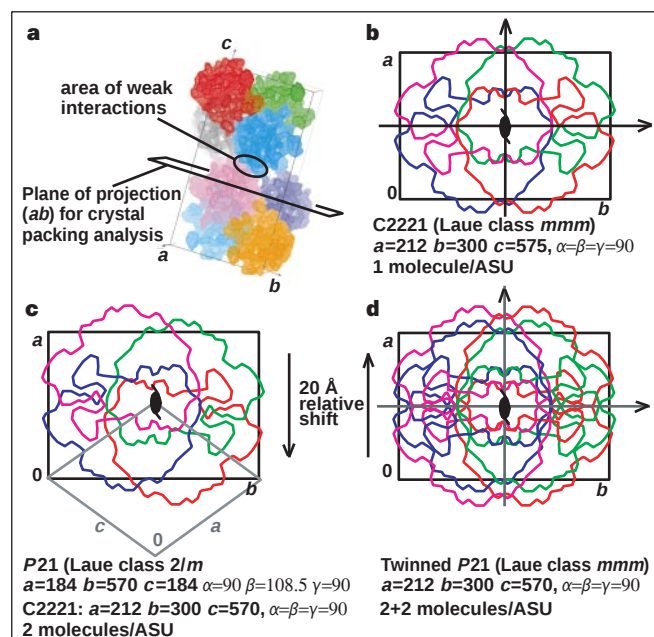


Figure 6 Packing diagrams of the 50S subunits in the orthorhombic, monoclinic and twinned monoclinic unit cells. **a**, Arrangement of eight 50S subunits (shown in red, orange, green and blue). **b–d**, Packing diagrams viewed down the **c** axis. The four extra subunits in the C222₁ unit cell that are translationally related to the subunits in the diagram are not shown for clarity. The monoclinic (**c**) cell results from the pairs of subunits (blue/green and red/orange) shifting by 10 Å from their positions in the orthorhombic (**b**) unit cell. The twinned crystal (**d**) reintroduces apparent two-fold symmetry, which is indicated with grey arrows.

have access to the cryo-electron microscopic maps¹⁹. Nevertheless, this model accounts for the protection of the SRL from modification that occurs when factors bind to the ribosome⁴¹.

The resulting models for factor binding predict several additional interactions between these G proteins and the 50S subunit. For example, the C-terminal domain of L6 is close to portions of the G domains of EF-G and EF-Tu, extensive contacts should occur between L14 and domains 2 and 3 of EF-G, and likewise between L14, EF-Tu and the acceptor stem of tRNA bound to EF-Tu. Thus, the model accounts for the observation that EF-G can be crosslinked to both L6 and L14 when it is bound to the ribosome in both the pre-translocation (using a non-hydrolysable GTP analogue) and post-translocation (with fusidic acid and GDP) states^{42,43}. Our present model does not, however, explain the ability of ribosome-bound EF-G to protect from modification the rRNA that interacts with L11 (ref. 41), or how it can be crosslinked to this rRNA⁴⁴ in both the pre- and post-translocation states⁴⁴. This inconsistency may indicate that the L11 assembly moves towards the EF-G factor when it binds—the gap between L11 and the SRL would allow such a movement.

Although it would be interesting to speculate on how the factor-binding centre stimulates the GTPase activity of EF-G and EF-Tu, our model with docked factors is based on biochemical data pertaining to a post-translocation state and our 50S subunit structure is in an unknown physiological state. That said, our model places the factor-bound GTP/GDP within contact distance of the SRL, and portions of their switch regions 1 and 2, which change conformation upon GTP hydrolysis, lie between protein L14 and the SRL. Arginine residues from L14, L6 and L11 are not located in the general vicinity of the GTP/GDP bound to either factor.

Summary

We have obtained a 5 Å-resolution map of the large ribosomal subunit in which proteins and rRNA segments of known structure

can be positioned, long stretches of rRNA backbone traced, and proteins of unknown structure located. The SRL, L6, L11, L14 and the 23S rRNA domain that binds L11, which together constitute most of the ribosome's factor-binding site, have all been located. The central position of the SRL in that site suggests it may stimulate the GTPase activity of factors like EF-G and EF-Tu when they are ribosome-bound. As data extending to 3 Å resolution can be obtained from these crystals, computation of a map at significantly higher resolution and its fitting by a complete atomic model of the 50S ribosomal subunit may soon be possible. □

Methods

Crystals. *H. marismortui* (ATCC 43049) was grown and 50S subunits prepared and crystallized as described^{21,46}. The C222₁ and merohedrally twinned P2₁ crystals grow under the same conditions and cannot be distinguished by gross morphology. C222₁ crystals with shorter *c* axis (540 Å) were obtained by increasing the concentration of PEG6000. Crystals were stabilized by gradual transfer into a solution containing ethylene glycol in addition to other components of the crystallization buffer, and flash-frozen in liquid propane. Heavy atom derivatives were prepared by soaking crystals in stabilization solutions containing the heavy atom compound of interest.

X-ray data collection and processing. All data were collected at the National Synchrotron Light Source (Brookhaven) from crystals frozen at 100 K, using beamlines X12b and X12c. For each heavy-atom derivative, anomalous diffraction data were recorded using either a 345-mm MAR imaging plate or a 2 × 2 CCD detector (Brancheis or Quantum). The beam size was 200 × 200 μm. Crystals were aligned along the long axis of the unit cell so that 1.0° oscillations could be used to collect reflections out to 3.2 Å resolution at the edge of the MAR detector. The crystal-to-detector distances varied between 450.0 mm and 550.0 mm depending on wavelength, crystal quality and beam divergence. Data sets were processed by using DENZO and SCALEPACK⁴⁷.

MIRAS phasing. The heavy-atom positions for the major binding sites were determined from combination difference anomalous and isomorphous Patterson maps at various resolution ranges, and minor sites and osmium hexamine sites were found by difference Fourier; heavy-atom cluster compounds and the refinement of their parameters have been described²¹. These phases were combined with SAD phases calculated using each derivative separately. All aspects of the isomorphous and anomalous phasing, including difference Patterson, difference Fourier map calculation and heavy-atom cluster rigid-body refinement against lack of closure, were done using the CNS program package⁴⁸.

Non-crystallographic averaging and detwinning. MIRAS/SAD combined phases were improved by intercrystal and non-crystallographic averaging between three different crystal forms: the C222₁ form, the twinned P2₁ form, and the C222₁ form with reduced *c* axis (Fig. 6). The merohedrally twinned data were detwinned at the level of intensities when the twinning fraction was below 35% and by back-transforming the map when the twinning was complete⁴⁹. As the second method of detwinning is more accurate when the phase estimates improve, it was combined with averaging and applied every ten cycles. Phases were improved by non-crystallographic and intercrystal averaging between four views of the large ribosomal subunit in three crystal forms using DMMULTI²² and RAVE²³ program packages. Several hundred cycles of averaging were performed starting from low-resolution reflections where average MIRAS/SAD figures of merit were greater than 0.5. The improved phases were combined with original MIRAS/SAD probabilities and subjected to solvent flipping using SOLOMON⁵⁰ as implemented in CNS⁴⁸. For the final map calculation, to compensate for the overestimation of phase accuracy, phase probabilities were 'blurred' by applying a 125 Å² temperature factor.

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1. Monro, R. E. Catalysis of peptide bond formation by 50S ribosomal subunits from *Escherichia coli*. *J. Mol. Biol.* **26**, 147–151 (1967).
2. Wittmann-Liebold, B., in *Structure, Function, and Genetics of Ribosomes* (eds Hardesty, B. & Kramer, G.) 326–361 (Springer, New York, 1986).
3. Frank, J. et al. A model for protein synthesis based on cryo-electron microscopy of the *E. coli* ribosome. *Nature* **376**, 441–444 (1995).
4. Stark, H. et al. Arrangement of tRNAs in pre- and posttranslocational ribosomes revealed by electron cryomicroscopy. *Cell* **88**, 19–28 (1997).

5. Milligan, R. A. & Unwin, P. N. T. Location of exit channel for nascent protein in 80S ribosome. *Nature* **319**, 693–695 (1986).
6. Beckmann, R. *et al.* Alignment of conduits for the nascent polypeptide chain in the ribosome–Sec61 complex. *Science* **278**, 2123–2128 (1997).
7. Liljas, A. in *The Ribosome. Structure, Function & Evolution* (eds Hill, W. E. *et al.*) 309–317 (Am. Soc. Microbiol., Washington DC, 1990).
8. Capel, M. S. *et al.* A complete mapping of the proteins in the small ribosomal subunit of *E. coli*. *Science* **238**, 1403–1406 (1987).
9. May, R. P., Nowotny, V., Nowotny, P., Voss, H. & Nierhaus, K. H. Inter-protein distances within the large subunit from *Escherichia coli* ribosomes. *EMBO J.* **11**, 373–378 (1992).
10. Oakes, M., Henderson, E., Scheinman, A., Clark, M. & Lake, J. A. in *Structure, Function and Genetics of Ribosomes* (eds Hardesty, B. & Kramer, G.) 47–67 (Springer, New York, 1986).
11. Stoeffer, G. & Stoeffer-Meilicke, M. in *Structure, Function, and Genetics of Ribosomes* (eds Hardesty, B. & Kramer, G.) 28–46 (Springer, New York, 1986).
12. Ramakrishnan, A. & White, S. W. Ribosomal protein structures: insights into the architecture, machinery and evolution of the ribosome. *Trends Biochem. Sci.* **23**, 208–212 (1998).
13. Szewczak, A. A. & Moore, P. B. The sarcin/ricin loop, a modular RNA. *J. Mol. Biol.* **247**, 81–98 (1995).
14. Correll, C. C. *et al.* Crystal structure of the ribosomal RNA loop essential for binding both elongation factors. *Proc. Natl Acad. Sci. USA* **95**, 13436–13441 (1998).
15. Correll, C. C., Freeborn, B., Moore, P. B. & Steitz, T. A. Metals, motifs and recognition in the crystal structure of a 5S rRNA domain. *Cell* **91**, 705–712 (1997).
16. Dallas, A. & Moore, P. B. The solution structure of the loop E/loop D region of *E. coli* 5S rRNA. *Structure* **5**, 1639–1653 (1997).
17. Wimberly, B. T., Guymon, R., McCutcheon, J. P., White, S. W. & Ramakrishnan, V. R. A detailed view of a ribosomal active site: the structure of the L11–RNA complex. *Cell* **97**, 491–502 (1999).
18. Conn, G. L., Draper, D. E., Lattman, E. E. & Gittis, A. G. Crystal structure of a conserved ribosomal protein RNA complex. *Science* **284**, 1171–1174 (1999).
19. Agrawal, R. K., Penczek, P., Grassucci, R. A. & Frank, J. Visualization of elongation factor G on the *Escherichia coli* 70S ribosome: the mechanism of translocation. *Proc. Natl Acad. Sci. USA* **95**, 6134–6138 (1998).
20. Stark, H. *et al.* Visualization of elongation factor Tu on the *Escherichia coli* ribosome. *Nature* **389**, 403–406 (1997).
21. Ban, N. *et al.* A 9 Å resolution X-ray crystallographic map of the large ribosomal subunit. *Cell* **93**, 1105–1115 (1998).
22. Cowtan, K. D. An automated procedure for phase improvement by density modification. *Joint CCP4 and ESF-EACBM Newsletter on Protein Crystallography* **31**, 34–38 (1994).
23. Jones, T. A. in *CCP4 Study Weekend, Molecular Replacement* (eds Dodson, E. J., Glover, S. & Wolf, W.) 91–105 (SSRC, Daresbury Laboratory, Warrington, Cheshire, UK, 1992).
24. Bernabeu, C. & Lake, J. A. Nascent polypeptide chains emerge from the exit domain of the large ribosomal subunit: Immune mapping of the nascent chain. *Proc. Natl Acad. Sci. USA* **79**, 3111–3115 (1982).
25. Kleywegt, G. J. & Jones, T. A. Template convolution to enhance or detect structural features in macromolecular electron-density maps. *Acta Crystallogr. D* **53**, 179–185 (1997).
26. Cate, J. *et al.* Crystal structure of a group I ribozyme domain: principles of RNA packing. *Science* **273**, 1678–1685 (1996).
27. Jucker, F. M., Heus, H. A., Yip, P. F., Moors, E. H. M. & Pardi, A. A network of heterogeneous hydrogen bonds in GNRA tetraloops. *J. Mol. Biol.* **264**, 968–980 (1996).
28. Stern, S., Powers, T., Changchien, L.-I. & Noller, H. F. RNA–protein interactions in 30S ribosomal subunits: folding and function of 16S rRNA. *Science* **244**, 783–790 (1989).
29. Golden, B. L., Ramakrishnan, V. & White, S. W. Ribosomal protein L6. Structural evidence of gene duplication from a primitive RNA binding protein. *EMBO J.* **12**, 4901–4908 (1993).
30. Leffers, H., Kjems, J., Ostergaard, L., Larsen, N. & Garrett, R. A. Evolutionary relationships amongst archaeobacteria: a comparative study of 23S ribosomal RNAs of a sulphur-dependent extreme thermophile, an extreme halophile, and a thermophilic mechanogen. *J. Mol. Biol.* **195**, 43–61 (1987).
31. Leffers, H., Egeberg, J., Andersen, A., Christensen, T. & Garrett, R. A. Domain VI of *Escherichia coli* 23S ribosomal RNA structure, assembly and function. *J. Mol. Biol.* **204**, 507–522 (1988).
32. Uchiyama, T., Sato, N., Wada, A. & Hachimori, A. Interaction of the sarcin/ricin domain of 23S ribosomal RNA with proteins L3 and L6. *J. Biol. Chem.* **274**, 681–686 (1999).
33. Urlaub, H., Kruft, V., Bischof, O., Müller, E. C. & Wittmann-Liebold, B. Protein–rRNA binding features and their structural and functional implications in ribosomes as determined by crosslinking studies. *EMBO J.* **14**, 4578–4588 (1995).
34. Davies, C., White, S. W. & Ramakrishnan, V. The crystal structure of ribosomal protein L14 reveals an important organizational component of the translational apparatus. *Structure* **4**, 55–66 (1996).
35. Walczek, J., Schuler, D., Stoeffer-Meilicke, M., Brimacombe, R. & Stoeffer, G. A model for the spatial arrangement of the proteins in the large subunit of the *Escherichia coli* ribosome. *EMBO J.* **7**, 3571–3576 (1988).
36. Nakagawa, A. *et al.* The three-dimensional structure of the RNA-binding domain of ribosomal protein L2: a protein at the peptidyl transferase center of the ribosome. *EMBO J.* **18**, 1459–1467 (1999).
37. Wilson, K. S. & Noller, H. H. Mapping the position of translational elongation factor EF-G in the ribosome by directed hydroxyl radical probing. *Cell* **92**, 131–139 (1998).
38. Aevsarsson, A. *et al.* Three-dimensional structure of the ribosomal translocase: elongation factor G from *Thermus thermophilus*. *EMBO J.* **13**, 3669–3677 (1994).
39. Czerwowski, J., Wang, J., Steitz, T. A. & Moore, P. B. The crystal structure of elongation factor G complexed with GDP at 2.7 Å resolution. *EMBO J.* **13**, 3661–3668 (1994).
40. Nissen, P. *et al.* Crystal structure of the ternary complex of Phe-tRNA^{Phe}, EF-Tu and a GTP analog. *Science* **270**, 1464–1472 (1995).
41. Moazed, D., Robertson, J. M. & Noller, H. F. Interaction of elongation factors EF-G and EF-Tu with a conserved loop in 23S RNA. *Nature* **334**, 362–364 (1988).
42. Traut, R. R. *et al.* in *Structure, Function, and Genetics of Ribosomes* (eds Hardesty, B. & Kramer, G.) 286–308 (Springer, New York, 1986).
43. Skold, S. E. Chemical crosslinking of elongation factor G to both subunits of the 70S ribosome from *E. coli*. *Eur. J. Biochem.* **127**, 225–229 (1982).
44. Skold, S. E. Chemical crosslinking of elongation factor G to the 23S rRNA in 70S ribosomes from *Escherichia coli*. *Nucleic Acids Res.* **11**, 4923–4932 (1983).
45. Nevskaya, N. *et al.* Structure of archaeal ribosomal protein L1 provides new insights into the RNA binding for the L1 protein family. *J. Mol. Biol.* (in the press).
46. von Bohlen, K. *et al.* Characterization and preliminary attempts for derivitization of crystals of large ribosomal subunits from *Haloarcula marismortui* diffracting to 3 Å resolution. *J. Mol. Biol.* **222**, 11–15 (1991).
47. Otwinowski, Z. in *Data Collection and Processing* (eds Sawyer, L., Isaacs, N. & Bailey, D.) 52–62 (SERC Daresbury Laboratory, Warrington, UK, 1993).
48. Brünger, A. T. *et al.* Crystallography and NMR system (CNS): a new software suite for macromolecular structure determination. *Acta Crystallogr. D* **54**, 905–921 (1998).
49. Yeates, T. O. Detecting and overcoming crystal twinning. *Methods Enzymol.* **276**, 344–358 (1997).
50. Abrahams, J. P. & Leslie, A. G. W. Methods used in the structural determination of bovine mitochondrial F1 ATPase. *Acta Crystallogr. D* **52**, 30–43 (1996).

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A radio pulsar with an 8.5-second period that challenges emission models

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Radio pulsars are rotating neutron stars that emit beams of radio waves from regions above their magnetic poles. Popular theories^{1–4} of the emission mechanism require continuous electron–positron pair production, with the potential responsible for accelerating the particles being inversely related to the spin period. Pair production will stop when the potential drops below a threshold, so the models predict that radio emission will cease when the period exceeds a value that depends on the magnetic field strength and configuration. Here we show that the pulsar J2144–3933, previously thought to have a period of 2.84 s, actually has a period of 8.51 s, which is by far the longest of any known radio pulsar. Moreover, under the usual model assumptions⁵, based on the neutron-star equations of state, this slowly rotating pulsar should not be emitting a radio beam. Therefore either the model assumptions are wrong, or current theories of radio emission must be revised.

PSR J2144–3933 was discovered in the Parkes Southern pulsar survey^{6,7}. In that survey, automated software determined its period, P , to be 2.84 s and subsequent observations and analysis of this pulsar were performed assuming this to be the correct period. In particular, timing observations were made for about four years from mid-1993 (ref. 8) and determined a period derivative of $(0.160 \pm 0.005) \times 10^{-15}$. This low value implied a large characteristic age ($\tau \approx 280$ Myr) and a moderately strong surface dipolar magnetic field ($B_s \approx 6 \times 10^{11}$ G).

In October 1994, as part of a programme to study pulse scintillation⁹, observations of this pulsar were made using the Parkes radio telescope in a continuous-sampling mode. The receiver was a dual-channel cooled system with a 32-MHz-wide band at

436 MHz. A filter-bank system with 256 channels across the 32 MHz was used to overcome the effects of interstellar dispersion. Data were recorded on PSR J2144–3933 at 1.2-ms intervals for ~ 45 min. An analysis of the fluctuations in pulse intensity in these data revealed that pulsed emission was present only every third pulse period. The obvious interpretation of this result is that the pulse period had been mis-identified in the original search process, and that the true pulse period is three times the nominal value, or 8.51 s. This is by far the longest period of any known radio pulsar—the previous longest¹⁰ was that of PSR J1951+1123, 5.09 s. Folding the data from PSR J2144–3933 at the 8.51-s period gives the mean pulse profile shown in Fig. 1. This shows the complete absence of pulsed emission at the one- and two-thirds points of the pulse profile, consistent with the identification of 8.51 s as the true period. Revised timing parameters are given in Table 1. We are currently checking if any other pulsars from this survey have mis-identified periods.

As a fraction of the pulse period, the mean pulse profile is extraordinarily narrow, by far the narrowest known, with a half-intensity width of less than one degree of longitude (Table 1), implying a similarly narrow beam of emission from the rotating neutron star. Such a narrow beam is consistent with the known inverse relationship between pulse period and beamwidth^{11,12}.

Observational studies^{11,13} have shown that pulse emission in the central or ‘core’ region of the pulse window has rather different

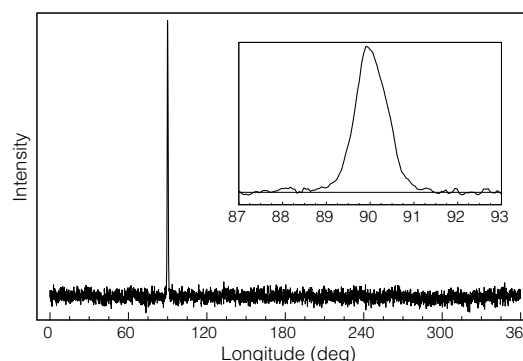


Figure 1 Pulse profile for PSR J2144–3933 at 436 MHz when folded with the correct period of 8.51 s. The sampling interval is 1.2 ms, but the profile has been smoothed with a three-point running mean. The horizontal axis is labelled in degrees of longitude, where 360° is the pulse period. The inset shows an expanded view of the region around the pulse.

Table 1 Parameters of PSR J2144–3933

Right ascension (J2000)	21 h 44 min 12.15(8) s
Declination (J2000)	−39° 33′ 54.89(12)″
Rotation period, P	8.5098274930(8) s
Period derivative, $\dot{P}$	$0.475(8) \times 10^{-15}$
Epoch of period (MJD)	49,016.0
Characteristic age, τ	2.81×10^8 yr
Profile width (436 MHz) at 10% of maximum, W_{10}	37 ms, 1.56°
Profile width (436 MHz) at 50% of maximum, W_{50}	20 ms, 0.84°
Mean flux density at 400 MHz, S_{400}	4 mJy
Mean luminosity at 400 MHz, L_{400}	0.13 mJy kpc ²
Spindown luminosity, $\dot{E}$	3.2×10^{28} erg s ^{−1}
Magnetic field strength, surface dipole component, B_s	2.0×10^{12} G

Timing parameters of PSR J2144–3933 obtained from a reanalysis of the 1993–97 timing data together with more recent data up to August 1998. Uncertainties in the last quoted digit are in parentheses and are twice the formal r.m.s. values given by the timing program. Concomitant with the increase in the period by a factor of three, the true period derivative is also a factor of three greater than that quoted in ref. 8. The surface dipole magnetic field strength, given by $B_s = 3.2 \times 10^{19} (P\dot{P})^{1/2}$ G, consequently is also increased by a factor of three. The characteristic age, given by $P/2\dot{P}$, remains unchanged with the new parameters. The observed¹⁴ value of W_{50} at 659 MHz (after compensating for the period misidentification) is 0.83° , which together with the width at 436 MHz, suggests a value of $\sim 0.82^\circ$ at 1 GHz. This is in good agreement with Rankin's¹² empirical relation for pulse width of core emission at a frequency of 1 GHz, which predicts a width of 0.84° assuming orthogonal rotation and magnetic axes. The agreement implies that the magnetic and rotation axes in this pulsar are not close to alignment, despite evidence for a tendency towards alignment in old pulsars²⁴.

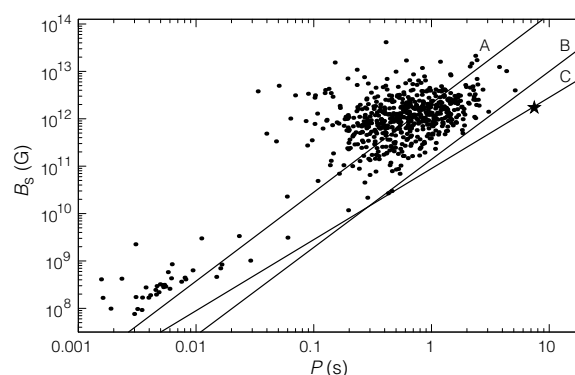


Figure 2 Distribution of known pulsars (excluding globular cluster pulsars) on the P – B_s plane, where P is the pulsar period and B_s is the surface magnetic dipole field strength. PSR J2144–3933 is marked with a filled star symbol. The sloping lines are ‘death lines’ according to various assumptions regarding field-line curvature in the emission region (see text). Line A, $4 \log B_s - 7.5 \log P = 49.3$; line B, $7 \log B_s - 13 \log P = 78$; line C, $4 \log B_s - 6 \log P = 43.8$.

properties from the emission in the outer or 'conal' regions. Polarization observations of this pulsar¹⁴ at 659 MHz show that the pulse is weakly polarized with a very rapid swing of position angle through the pulse. This, and the narrow and nearly gaussian pulse profile, suggests that the entire observed pulse is core-type emission with the beam direction close to that of the magnetic axis.

We note that the period of PSR J2144–3933 is similar to the periods of anomalous X-ray pulsars¹⁵ (AXPs) and pulsars identified with soft γ -ray repeaters¹⁶ (SGRs). These slowly spinning neutron stars have been interpreted as 'magnetars', whose emission is powered not by the rotational kinetic energy of the neutron star, but by the decay of its super-strong magnetic field¹⁷. No AXPs or SGRs have yet been detected as radio pulsars, and it has been argued that such pulsars may not emit in the radio band because photon splitting inhibits pair-production¹⁸. However, we note that the lack of detection of radio emission from the few known AXPs and SGRs can be explained if their radio beamwidths are similar to that of PSR J2144–3933; if this were so, only a very small fraction of the total population of such objects would be visible from Earth as radio pulsars. The surface magnetic field of PSR J2144–3933 itself is much less than that of magnetars, and no magnetar-like X-ray emission would be expected from this object.

For a given magnetic field configuration, the locus of P – B_s values at which radio emission ceases defines a 'death line' in the P – B_s diagram (Fig. 2), to the right of which no pulsar should emit radio waves. By invoking a variety of assumptions about field-line curvature in the emission region above the neutron star, Chen and Ruderman¹⁹ derived a set of death lines. For a given surface magnetic dipole field strength, pulsars with strong multipolar field components will have a highly curved field near the stellar surface permitting the emission to persist to longer periods. In Fig. 2, death line B corresponds to the greatest reasonable magnetic field curvature, and death line C is an extreme and unlikely case. No pulsar should exist with a surface magnetic dipole field strength and period which place it to the right of these death lines. PSR J2144–3933 lies to the right of both lines. It is (to our knowledge) the first pulsar known to do so, and calls into question the assumptions made in deriving the death lines.

One possibility is that the neutron-star properties might differ from those commonly assumed. For example, the derived value of B_s is proportional to $(I/R^6)^{1/2}$, where I and R are the neutron-star moments of inertia and radius, respectively⁵. For different equations of state, I/R^6 may vary by up to two orders of magnitude. If PSR J2144–3933 has a larger-than-average value of I/R^6 , the magnetic dipole field strength may be large enough to permit pair production and hence radio emission.

Alternatively, it may be that the radio emission process does not depend on pair-production. Weatherall and Eilek²⁰ have suggested that pulsars lying below line A (Fig. 2) all have conal properties, whereas most of those above it are dominated by core emission; they also suggested that conal emission may be generated by a mechanism not dependent on pair production. PSR J2144–3933 has clear signatures of core emission, and the presence of this pulsar beyond death line A is inconsistent with the hypothesis of Weatherall and Eilek. We note that PSR J1951+1123 also lies well below this death line and has a very narrow pulse, consistent with expectations for core emission^{10,12}. We suggest that the fact that few core-dominated pulsars are seen beyond death line A is a selection effect due to the narrow beamwidth of the core emission.

PSR J2144–3933 has the lowest spindown luminosity ($\sim 3 \times 10^{28}$ erg s^{−1}) of any known pulsar. It is also relatively nearby—the distance, d , estimated from the dispersion measure^{8,21} is only ~ 180 pc—and so its radio luminosity, L_{400} , is low; $L_{400} = S_{400} d^2 \approx 0.13$ mJy kpc², where S_{400} is its mean flux density at 400 MHz. If the beam is assumed to be circular, the beaming fraction (that is, the fraction of the celestial sphere swept across by the beam) is also extremely small, ~ 0.01 . Its low luminosity and

the very low beaming fraction together imply that we can observe only a very small proportion of the total population of such objects in the Galaxy. While extrapolation from the detection of a single object is always uncertain (some would say foolhardy), there is no reason to suppose that PSR J2144–3933 is unique. With this caveat, this detection implies a Galactic population of similar pulsars of the order of 10^5 , comparable to previous estimates of the size of the total pulsar population^{22,23}. □

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1. Ruderman, M. A. & Sutherland, P. G. Theory of pulsars: Polar gaps, sparks, and coherent microwave radiation. *Astrophys. J.* **196**, 51–72 (1975).
2. Machabeli, G. Z. & Usov, V. V. Cyclotron instability in the magnetosphere of the Crab Nebula pulsar, and the origin of its radiation. *Sov. Astron. Lett.* **5**, 238–241 (1979).
3. Cheng, A. F. & Ruderman, M. A. Particle acceleration and radio emission above pulsar polar caps. *Astrophys. J.* **235**, 576–586 (1980).
4. Beskin, V. S., Gurevich, A. V. & Istomin, Y. N. Theory of the radio emission of pulsars. *Astrophys. Space Sci.* **146**, 205–281 (1988).
5. Manchester, R. N. & Taylor, J. H. *Pulsars* (Freeman, San Francisco, 1977).
6. Manchester, R. N. *et al.* The Parkes Southern Pulsar Survey—I. Observing and data analysis systems and initial results. *Mon. Not. R. Astron. Soc.* **279**, 1235–1250 (1996).
7. Lyne, A. G. *et al.* The Parkes Southern Pulsar Survey—II. Final results and population analysis. *Mon. Not. R. Astron. Soc.* **295**, 743–755 (1998).
8. D'Amico, N. *et al.* The Parkes Southern Pulsar Survey—III. Timing of long-period pulsars. *Mon. Not. R. Astron. Soc.* **297**, 28–40 (1998).
9. Johnston, S., Nicastro, L. & Koribalski, B. Scintillation parameters for 49 pulsars. *Mon. Not. R. Astron. Soc.* **297**, 108–116 (1998).
10. Camilo, F. & Nice, D. J. Timing parameters of 29 pulsars. *Astrophys. J.* **445**, 756–761 (1995).
11. Lyne, A. G. & Manchester, R. N. The shape of pulsar radio beams. *Mon. Not. R. Astron. Soc.* **234**, 477–508 (1988).
12. Rankin, J. M. Toward an empirical theory of pulsar emission. IV. Geometry of the core emission region. *Astrophys. J.* **352**, 247–257 (1990).
13. Rankin, J. M. Toward an empirical theory of pulsar emission. I. Morphological taxonomy. *Astrophys. J.* **274**, 333–358 (1983).
14. Manchester, R. N., Han, J. L. & Qiao, G. J. Polarization observations of 66 southern pulsars. *Mon. Not. R. Astron. Soc.* **295**, 280–298 (1998).
15. van Paradijs, J., Taam, R. E. & van den Heuvel, E. P. J. On the nature of the 'anomalous' 6-s X-ray pulsars. *Astron. Astrophys.* **299**, L41–L44 (1995).
16. Kouveliotou, C. *et al.* An X-ray pulsar with a superstrong magnetic field in the soft γ -ray repeater SGR1806–20. *Nature* **393**, 235–237 (1998).
17. Duncan, R. C. & Thompson, C. Formation of very strongly magnetized neutron stars: Implications for gamma-ray bursts. *Astrophys. J.* **392**, L9–L13 (1992).
18. Baring, M. G. & Harding, A. K. Radio-quiet pulsars with ultra-strong magnetic fields. *Astrophys. J.* **507**, L55–L58 (1998).
19. Chen, K. & Ruderman, M. Pulsar death lines and death valley. *Astrophys. J.* **402**, 264–270 (1993).
20. Weatherall, J. C. & Eilek, J. A. Are there two pulsar emission mechanisms? *Astrophys. J.* **474**, 407–413 (1997).
21. Taylor, J. H. & Cordes, J. M. Pulsar distances and the Galactic distribution of free electrons. *Astrophys. J.* **411**, 674–684 (1993).
22. Lyne, A. G., Manchester, R. N. & Taylor, J. H. The Galactic population of pulsars. *Mon. Not. R. Astron. Soc.* **213**, 613–639 (1985).
23. Lorimer, D. R., Bailes, M., Dewey, R. J. & Harrison, P. A. Pulsar statistics: The birthrate and initial spin periods of radio pulsars. *Mon. Not. R. Astron. Soc.* **263**, 403–415 (1993).
24. Tauris, T. M. & Manchester, R. N. On the evolution of pulsar beams. *Mon. Not. R. Astron. Soc.* **298**, 625–636 (1998).

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Measurement of gravitational acceleration by dropping atoms

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Laser-cooling of atoms and atom-trapping are finding increasing application in many areas of science¹. One important use of laser-cooled atoms is in atom interferometers². In these devices, an atom is placed into a superposition of two or more spatially separated atomic states; these states are each described by a quantum-mechanical phase term, which will interfere with one another if they are brought back together at a later time. Atom

interferometers have been shown to be very precise inertial sensors for acceleration^{3,4}, rotation⁵ and for the measurement of the fine structure constant⁶. Here we use an atom interferometer based on a fountain of laser-cooled atoms to measure g , the acceleration of gravity. Through detailed investigation and elimination of systematic effects that may affect the accuracy of the measurement, we achieve an absolute uncertainty of $\Delta g/g \approx 3 \times 10^{-9}$, representing a million-fold increase in absolute accuracy compared with previous atom-interferometer experiments⁷. We also compare our measurement with the value of g obtained at the same laboratory site using a Michelson interferometer gravimeter (a modern equivalent of Galileo's 'leaning tower' experiment in Pisa). We show that the macroscopic glass object used in this instrument falls with the same acceleration, to within 7 parts in 10^9 , as a quantum-mechanical caesium atom.

Here we focus on extending the precision of atom interferometers by increasing the interferometer measurement time with the use of an atomic fountain of laser-cooled atoms and by using atom optics components based on optical pulses of light. We have shown⁴ how an atom interferometer may be used to measure g with a precision of 1 part in 10^{10} . The absolute relative uncertainty $\Delta g/g$ of 3 parts in 10^9 achieved in the current work demonstrates that this type of atom interferometer can be used to make absolute measurements comparable with the most sensitive measurement tools in physics. We believe that this work is also the best confirmation of the equivalence principle between a quantum and macroscopic object. By comparison, there still remains a discrepancy of a few per cent between g measured by neutron interferometry and a macroscopic object⁸, and we may conclude that there are aspects of neutron interferometry that are not well understood. Finally, the high fringe contrast seen in this interferometer places severe constraints on speculations concerning the possibility of quantum-state phase diffusion due to space-time fluctuations⁹.

Our atom interferometer uses optical pulses of light to stimulate transitions between two different states of the atoms. The atoms are first exposed to an optical ' $\pi/2$ -pulse' defined as a pulse of light that puts an atom initially in the state $|1, p\rangle$, characterized by an internal state $|1\rangle$ and momentum p , into an equal superposition of the original state $|1, p\rangle$ and a second state; this second state is characterized by an internal state $|2\rangle$ and momentum $p + \hbar k$, and is denoted

by $|2, p + \hbar k\rangle$. Here, $\hbar k$ is the momentum imparted by the optical pulse. After a time T , the two parts of the atom drift apart by a distance $\hbar k T / M$, where M is the mass of the atom. Excitation by a so-called ' π -pulse' induces the part of the atom in state $|1, p\rangle$ to make the transition $|1, p\rangle \rightarrow |2, p + \hbar k\rangle$ and the part of the atom in $|2, p + \hbar k\rangle$ to make the transition $|2, p + \hbar k\rangle \rightarrow |1, p\rangle$. After another time T , the two parts of the atom come back together and a second $\pi/2$ -pulse with the appropriate phase relative to the atomic phase can put the atom into either of the states $|1, p\rangle$ or $|2, p + \hbar k\rangle$. Earlier analysis³ has shown that the phase difference between the two paths of the interferometer is given by

$$\Delta\Phi = (\Phi_1(t_1) - \Phi_2(t_2))_{\Gamma_1} - (\Phi_2(t_2) - \Phi_3(t_3))_{\Gamma_2}$$

where $\Phi_i(t_i) = k z_i - \omega t_i$ are the phases of the laser light at positions z_i and times t_i at the beginning of each optical pulse, ω is the frequency of the light, and Γ_i denotes the two classical paths. In our experiment, the frequency of the light is changed in a phase-continuous way, so that it remains resonant with the transition $|1, p\rangle \rightarrow |2, p + \hbar k\rangle$ as the atom accelerates owing to gravity. Under these conditions, we have shown³ that $\Delta\Phi = kgT^2$.

The internal states used in the experiment are the two magnetic-field-insensitive hyperfine ground states of caesium. Counter-propagating laser beams induce two-photon Raman transitions between these states, which doubles the mechanical effect of a single-photon transition. More importantly, the optical transition is determined by the frequency difference of two laser beams which is phase-locked to a stable microwave source. Thus, we have precise control of the frequency and the phase of the light.

We have analysed the complications due to gravity. The lowest-order correction to g for a constant gravity gradient γ is

$$\Delta g = \gamma \left(\frac{7}{12} g_0 T^2 - v_0 - z_0 \right)$$

where z_0 and v_0 are the position and velocity of the atom referenced to a point in the laboratory free of vibrations just before the first $\pi/2$ -pulse¹⁰. In our laboratory, $\gamma \approx 3 \times 10^{-7} \text{ g m}^{-1}$, due mostly to the gradient of the Earth's gravitational field. For typical experimental parameters, the gradient correction is 31 parts per billion (p.p.b.).

The experimental set-up is shown in Fig. 1. About 5×10^8 caesium atoms are extracted from a low-pressure background vapour and loaded into a magneto-optic trap (MOT) in 600 ms. After turning off the magnetic fields, the atoms are launched using moving polarization gradient optical molasses¹¹. During this time,

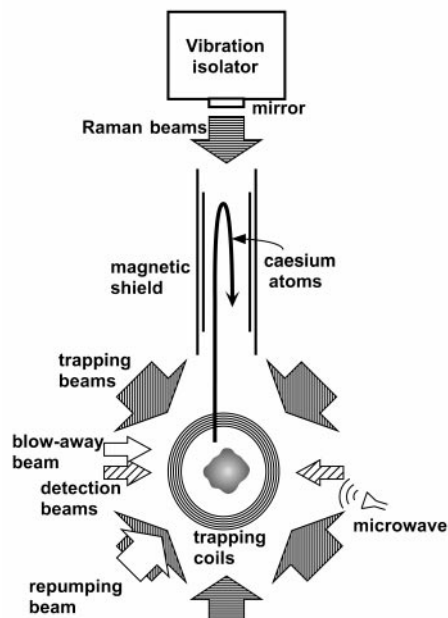


Figure 1 Overview of the experimental set-up.

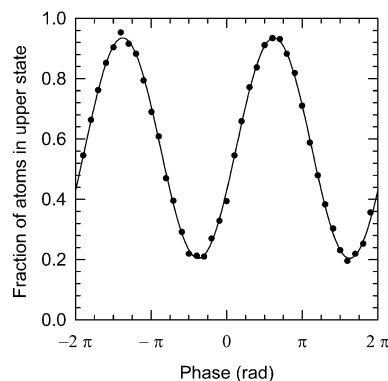


Figure 2 Typical Doppler-sensitive interferometer fringe for $T = 160$ ms. Shown are the 588,638th and 588,639th fringes. Each of the 40 data points represents a single launch of the atoms, spaced 1.3 s apart and taken over a period of 1 min. One full fringe corresponds to $\sim 2 \times 10^6 g$. Performing a least-squares fit determines local gravity to approximately $3 \times 10^{-9} g$.

the atoms are further cooled by tuning the molasses beams 60 MHz below resonance and lowering the intensity to 5 mW cm^{-2} . In the final stages of the launch, laser intensities are decreased to zero in $400 \mu\text{s}$ so that the atoms are also adiabatically cooled¹². The final temperature of the launched atoms is $\sim 1.5 \mu\text{K}$.

The launched atoms are subjected to a sequence of pulses (microwave, velocity-selective Raman, and state-selective blow-away) that places 3×10^6 atoms in the $6S_{1/2}$, $F = 3$, $m_F = 0$ state with an effective vertical temperature of 10 nK. This low velocity spread gives us a fringe contrast of $\sim 65\%$ for all times T between 0.5 and 160 ms.

The interferometer measurement occurs in a magnetically shielded region. The optical pulses are derived from two external-cavity phase-locked diode lasers. Different polarization configurations and intensities have been used throughout the experiment without affecting the accuracy or sensitivity of the measured g , within our experimental relative uncertainty of 2 p.p.b. The Raman difference frequency is controlled by a direct digital frequency synthesizer which is referenced to a Loran-C frequency standard. The frequency difference is switched between three fixed frequencies to compensate for the gravity-induced Doppler shift during the 320-ms interferometer free-fall time¹⁰.

Both Raman beams enter the vacuum chamber from below and are retro-reflected (Fig. 1). As the atomic transitions are Doppler sensitive, only one beam from each pair of upward- and downward-travelling beams is in resonance with the atoms. Because the two Raman beams travel over a common path before entering the vacuum system, laboratory vibrations frequency-shift the two beams by nearly the same amount. Thus, only vibrations of the

Table 1 Known noise sources

Noise source	σ_g (p.p.b.)
Atom shot noise	0.16
Intensity and frequency fluctuation of detection laser	0.9
Loran-C frequency stability	3.0
Raman-laser intensity noise	3.5
Residual vibrations	5
Fluctuation of fluorescence with no MOT atoms	7
Phase noise of 9.2-GHz source	11
Overall known noise sources	15
Observed noise	19

Known noise sources and their estimated effect on a gravity measurement with $T = 160$ ms between interferometer pulses of lengths $\tau_e = 80 \mu\text{s}$ and $\tau_{\text{avg}} = 40 \mu\text{s}$. σ_g denotes the standard deviation of a set of 40 measurements. If the actual vibration noise of the platform is a factor of 2 higher than noise calculated from the error signal, we have a full accounting of the observed noise.

retro-mirror affect the Raman difference frequency. This mirror is mounted on an actively stabilized, vibration isolation system. The acceleration, measured by a low-noise, low-frequency force feedback accelerometer (CMG-3V, Guralp, Reading, UK), is digitized and processed. An output signal is then used to control the current through a solenoid actuator. The platform has an effective resonance of 0.02 Hz and reduces vibrations between 0.2 to 5 Hz by two orders of magnitude¹³. Without this vibration isolation, our interference fringes vanish for T greater than ~ 40 ms. With the feedback circuit on, there is essentially no loss of fringe contrast up to the maximum drop times, limited by the size of the magnetically shielded region. Further motion control in our apparatus includes an active tilt control to maintain the tilt of the retro-mirror to within $10 \mu\text{rad}$. Rotations of the optical table about the horizontal axes are reduced through three one-dimensional active feedback vibration isolators placed at three corners of the table.

Typical interferometer data for a pulse spacing of $T = 160$ ms are shown in Fig. 2. One minute of integration time allows us to determine g with a precision of $3 \times 10^{-9} g$. To locate the central fringe, we vary T from 2 to 160 ms. Figure 3a shows a continuous measurement of g over a period of 3 days, with each dot corresponding to data similar to Fig. 2. Figure 3b shows the difference between our measurement and two tidal models¹⁴. The tidal model that included the ocean loading¹⁵ effect on our local environment is found to agree with our data with a precision of $1 \times 10^{-10} g$ after two days of integration time.

During the course of our experiment, we have arranged for an absolute gravimeter¹⁶ (FG-S, Micro-g Solutions, Arvada, Colorado), to be run in our laboratory for three days to measure the absolute value of g . This is a Michelson optical interferometer with one arm defined by a freely falling corner-cube and has a quoted relative uncertainty of 2 p.p.b. A comparison with the value of g we obtained in a 2-day run shows a difference of (7 ± 7) p.p.b. This comparison was limited mostly by a 5-p.p.b. uncertainty in our measurement of the local gravity gradient, which produces a 3 p.p.b. correction per cm vertical displacement. The two instruments were separated by 2 metres in our laboratory with a 0.5-m height difference.

Direct comparison of the noise of both instruments also showed that our atom interferometer has a 4 times higher resolution than the falling-corner-cube gravimeter, mainly due to the slower FG5 repetition rate (1/15 Hz) compared with our instrument (1/1.3 Hz). The noise per launch for both instruments was similar.

The use of an active low-frequency vibration isolator overcomes the main limitation of the previous g experiment. With a well supported vibration isolator enclosed in an acoustic isolation box and proper alignment of all the components of the system with the atoms, the noise due to vibrations, calculated from an integration of the power spectral density of the noise and the frequency-dependent system response, was found to be 5 p.p.b. This is lower than some of the other main noise sources (Table 1). With better frequency

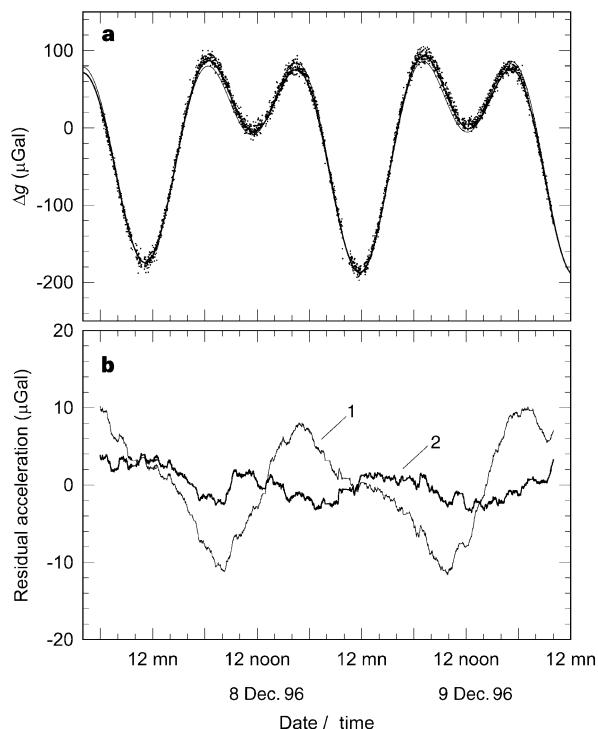


Figure 3 Comparison between experimental data and tide models. **a**, A closer look at two days of gravity data. Each data point represents a one-minute gravity measurement. The solid lines represent two different tidal models. $1 \mu\text{Gal} = 10^{-8} \text{ m s}^{-2} \approx 10^{-9} g$. **b**, the residuals of the data with respect to a tidal model where (trace 1) the Earth is modelled as a solid elastic object and where (trace 2) the effects of ocean loading of the Earth are taken into account. Data for ocean loading were provided by H.-G. Scherneck. Effects at the few p.p.b. level, such as changes in the local barometric pressure, have not been included.

Table 2 The main known potential systematic effects

	Relative uncertainty (p.p.b.)
Systematic error	
Cs wavelength	0.3
Laser lock offset	0.4
r.f. phase shift	2
Coriolis effect	2
Gravity gradient	0.2
a.c. Stark shift	1
Dependence on pulse timing	1
Overall instrumental uncertainty	3.2
Environmental effect	
Pressure correction	1
Ocean loading	1
Other environmental effects	2

Systematic effects that are ≤ 0.1 p.p.b. are not listed: these include possible effects of magnetic field gradients. The environmental effects are important in comparing values of g obtained at different times. Other environmental effects include water-table correction.

sources, active stabilization of the intensity of the Raman beams, and moving the detection region away from the caesium beam, these main noises could be reduced to less than 1 p.p.b. Further investigation of an artificial noise floor of the vibration isolator system could bring the eventual performance of the instrument to within a factor of 10 of the shot-noise limit.

Table 2 shows the most important systematic effects that we have identified, and their associated uncertainties. Other effects, such as magnetic-field gradients, wavefront curvature, speckle, dispersion in the air and windows, timing and switching errors in the optical pulses, were experimentally found to be below the 0.1-p.p.b. level and are not listed. We have also estimated relativistic corrections (<0.1 p.p.b.) and the effect of a changing effective wavevector due to different propagation delays during the interferometer sequence (corrected to an uncertainty of <0.1 p.p.b.). We now consider the possible contributions of other potential sources of instrumental uncertainty.

Dynamical diffraction treatment in neutron interferometer experiments corrected the original measurement of g by a few per cent (ref. 8). Borde pointed out that a similar effect is also present in our experiment, and should depend on the detuning of the laser from the atomic resonance caused by gravitational acceleration during the pulse¹⁷. Experimentally, we found no difference (within the experimental uncertainty of 2 p.p.b.) whether the detuning was always kept at zero by chirping the pulse to account for the acceleration due to gravity or whether detuning was zero only at the middle of the pulse. Furthermore, the effect of a common detuning on all three pulses was measured to be 1.3 p.p.b. for every Kilohertz detuning from the resonance condition. As the detuning in our experiment is less than 100 Hz, we estimate that this effect should be less than 1 p.p.b. for our experiment.

The light pulses could potentially induce a.c. Stark shifts and lead to systematic error. We measure the a.c. Stark shift by introducing an off-resonance Raman pulse that does not induce transitions in the time between the interferometer $\pi/2$ and π -pulses. This shift can be made less than 1 p.p.b. by adjusting the ratio of the Raman beam intensities. However, as this ratio tends to fluctuate during the long runs, we assign a 1 p.p.b. uncertainty to this effect.

Coriolis effects occur when atoms have a horizontal velocity. In this case, the photon recoils during the interferometer pulses are not parallel to the atomic velocity, and cause the atom interferometer to enclose a spatial area. The resulting sensitivity to rotation shifts the measured gravity by $\Delta g = 2\Omega \cdot (\mathbf{v}_0 \times \hat{\mathbf{k}})$, where Ω is the angular rotation, $\mathbf{v}_0$ is the velocity of the atom and $\hat{\mathbf{k}}$ is the direction of the photon recoil. At the latitude of our laboratory, 37.4° N, the Earth's rotation of $7.25 \times 10^{-5} \text{ rad s}^{-1}$ would thus cause a 1 p.p.b. shift in g for atoms that have a velocity of 0.0087 cm s^{-1} in the east–

west direction, which is small compared to the typical velocity spread of the atomic cloud ($\sim 1.5 \text{ cm s}^{-1}$). This effect can be reduced to zero if we choose a detection region that equalizes the number of atoms falling to the east and west. By rocking the laser table, and thereby introducing large rotations, we are able to locate this position to within 0.01 cm, resulting in a relative uncertainty of 2 p.p.b. in g .

Nonlinear frequency-dependent phase shifts in the radio-frequency system controlling the Raman difference frequency mimic a gravity signal. We have substantially reduced this effect by correcting for measured phase shifts, and by averaging results for different directions of $\mathbf{k}$. However, these procedures are difficult, and the remaining relative uncertainty of 2 p.p.b. is still one of the leading systematic effects.

We have measured the gravity gradient in the interaction region by launching the atoms to progressively lower heights using $T = 90 \text{ ms}$ fringes to stay within the magnetically shielded region. The values of g at different heights show that the gradient is constant and equal to the free-air gradient. This justifies the use of the formula assuming a constant gradient.

We have varied the time of the $\pi/2$ – π – $\pi/2$ pulses relative to the time of the launch. We observe a variation in g that quantitatively agrees with a calculated change due to the gravity gradient and the change in the magnitude of the $\mathbf{k}$ -vectors of the light. The fit of our data to the calculated curve (residual ≈ 1 p.p.b.) allows us to set an upper limit to a systematic effect due to any 'trajectory effect'.

At p.p.b. levels, uncertainties due to environmental effects become significant. The main uncertainties are listed in Table 2. However, these effects are not part of the instrumental uncertainty.

The measurement that we report here represents a million-fold increase in absolute accuracy compared to measurements obtained by previous atom interferometers. From our study of the systematic effects of our measurement, we consider that further improvements in the control of Coriolis effect—by, for example, rotating the whole system—and ensuring that there is no synchronized noise in the vibration isolator should lead to a relative uncertainty of the order of one part in 10^{10} . □

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1. Chu, S., Cohen-Tannoudji, C. & Phillips, W. D. Nobel lectures in physics 1997. *Rev. Mod. Phys.* **70**, 685–741 (1998).
2. Berman, P. R. (ed.) *Atom Interferometry* (Academic, Chestnut Hill, 1997).
3. Kasevich, M. & Chu, S. Measurement of the gravitational acceleration of an atom with a light pulse atom interferometer. *Appl. Phys. B* **54**, 321–332 (1992).
4. Peters, A., Chung, K. Y., Young, B., Hensley, J. & Chu, S. Precision atom interferometry. *Phil. Trans. R. Soc. Lond. A* **355**, 2223–2234 (1997).
5. Gustavson, T. L., Bouyer, P. & Kasevich, M. Precision rotation measurements with an atom interferometer gyroscope. *Phys. Rev. Lett.* **78**, 2046–2049 (1997).
6. Young, B. *A measurement of fine-structure constant using atom interferometry*. Thesis, Stanford Univ. (1997).
7. Ekstrom, C. R., Schmiedmayer, J., Chapman, M. S., Hammond, T. D. & Pritchard, D. E. Measurement of electric polarizability of sodium with an atom interferometer. *Phys. Rev. A* **51**, 3883–3888 (1995).
8. Werner, S. A. Quantum phase shifts induced by gravity and rotation. *J. Phys. Soc. Jpn* **65**, 51–59 (1997).
9. Percival, I. & Strunz, W. Detection of space-time fluctuations by a model matter interferometer. *Proc. R. Soc. Lond. A* **453**, 431–446 (1997).
10. Peters, A. *High precision gravity measurements using atom interferometry*. Thesis, Stanford Univ. (1998).
11. Weiss, D. S., Riis, E., Kasevich, M., Moler, K. A. & Chu, S. in *Light Induced Kinetic Effects on Atoms, Ions and Molecules* (eds Moi, I., Gozzini, S., Gabbanini, C., Arimondo, E. & Strumia, F.) 35–44 (ETS Editrice, Pisa, 1991).
12. Kastberg, A., Phillips, W. D., Rolston, S. L., Spreew, R. J. C. & Jessen, P. Adiabatic cooling of cesium to 700 nK in an optical lattice. *Phys. Rev. Lett.* **74**, 1542–1545 (1995).
13. Hensley, J. M., Peters, A. & Chu, S. Active low frequency vertical vibration isolation. *Rev. Sci. Instrum.* **70**, 2735–2741 (1999).
14. Melchior, P. J. *The Tides of Planet Earth* (Pergamon, Oxford, 1983).
15. Scherneck, H. G. A parameterized solid Earth tide model and ocean tide loading effects for global geodetic baseline measurements. *Geophys. J. Int.* **106**, 677–694 (1991).
16. Niebauer, T. N., Sasagawa, G., Faller, J. & Hilt, R. A new generation of absolute gravimeters. *Metrologia* **32**, 159–180 (1995).
17. Borde, C. J. in *Atom Interferometry* (ed. Berman, P. R.) 257–292 (Academic, Chestnut Hill, 1997).

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Thermally induced ultrasonic emission from porous silicon

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The most common mechanism¹ for generating ultrasound in air is via a piezoelectric transducer, whereby an electrical signal is converted directly into a mechanical vibration. But the acoustic pressure so generated is usually limited to less than 10 Pa, the frequency bandwidth of most piezoelectric ceramics is narrow, and it is difficult to assemble such transducers into a fine-scale phase array with no crosstalk^{2,3}. An alternative strategy using micromachined electrostatic diaphragms is showing some promise^{4,5}, but the high voltages required and the mechanical weakness of the diaphragms may prove problematic for applications. Here we show that simple heat conduction from porous silicon to air results in high-intensity ultrasound without the need for any mechanical vibrational system. Our non-optimized device generates an acoustic pressure of 0.1 Pa at a power consumption of 1 W cm⁻², and exhibits a flat frequency response up to at least 100 kHz. We expect that substantial improvements in efficiency should be possible. Moreover, as this material lends itself to integration with conventional electronic circuitry, it should be relatively straightforward to develop finely structured phase arrays of these devices, which would give control over the wavefront of the acoustic emissions.

The idea of a thermal sound generator (a "thermophone") was proposed 80 years ago⁶, in which the acoustic element was a simple self-supporting, thin metal film. The photoacoustic effect in porous silicon has also been reported^{7,8}, but was used to characterize the porous silicon itself from gas expansion in a closed space. One might think that ultrasound generation by heat exchange is not possible, as

the thermal conduction is too slow. However, we report here that the experimental device shown in Fig. 1 operates as an efficient ultrasound emitter. It is composed of a patterned, thin aluminium film electrode (30 nm thick), a microporous silicon layer (10 µm thick), and a p-type crystalline silicon (c-Si) wafer. The porous silicon layer consists of many confined silicon nanocrystallites with three-dimensional nanopores⁹. In our experiment, the porous silicon layer (with porosity of ~70%) was formed by a conventional anodization technique in a solution of 55% HF:ethanol = 1:1 at a temperature of 20 °C at a current density of 20 mA cm⁻² for 8–40 min. The aluminium electrode was used to input a sinusoidal current into the porous silicon layer, the temperature of which was raised by Joule's heating. The emitted acoustic pressure was measured as a function of output frequency by a microphone type 4138, Brüel & Kjær, Denmark) placed at a position of 3.5 cm from the front surface.

We now explain the device concept, based on a theoretical analysis of thermal conduction phenomena¹⁰ in the porous-silicon/air system as illustrated in Fig. 2a. Suppose that a thermal power density $q(\omega)\exp(j\omega t)$ (in units of W cm⁻²) with an angular frequency ω is provided at the surface of the porous silicon layer through a sufficiently thin metal film on it. When the thickness d of the porous silicon layer is such that

$$d > D \equiv \sqrt{\frac{2\alpha}{\omega C}} \quad (1)$$

where α and C are respectively the thermal conductivity and heat capacity per unit volume of porous silicon, then the surface temperature change $T_0(\omega)\exp(j\omega t)$ is given by

$$T_0(\omega) = \frac{q(\omega)}{\sqrt{j\omega\alpha C}} \quad (2)$$

if the expansion of porous silicon and the heat flow into the air are neglected (see Fig. 2b). The temperature change induces an acoustic pressure $P(x, \omega)\exp(j\omega t)$ through the alternating thermal expansion of the air. Using the fundamental equations of photoacoustic analysis¹¹, we find that

$$P(x, \omega) = A \frac{\exp(-jkx)}{\sqrt{\alpha C}} q(\omega), \quad A = \sqrt{\frac{\gamma\alpha_a}{C_a}} \frac{P_A}{vT_A} \quad (3)$$

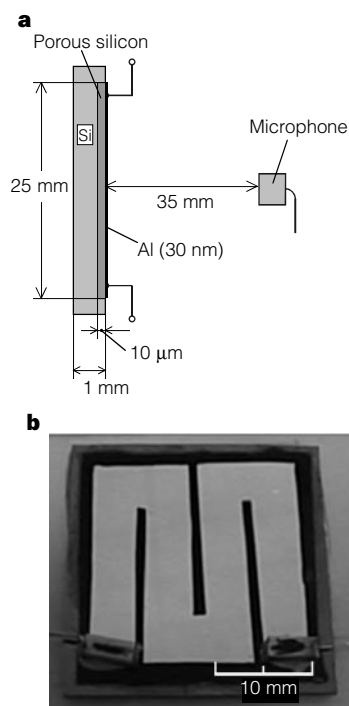


Figure 1 Our device for producing thermally induced ultrasonic emission. **a**, Cross-sectional view of the fabricated device and set-up for sound measurement. **b**, Photograph of a top view of our device.

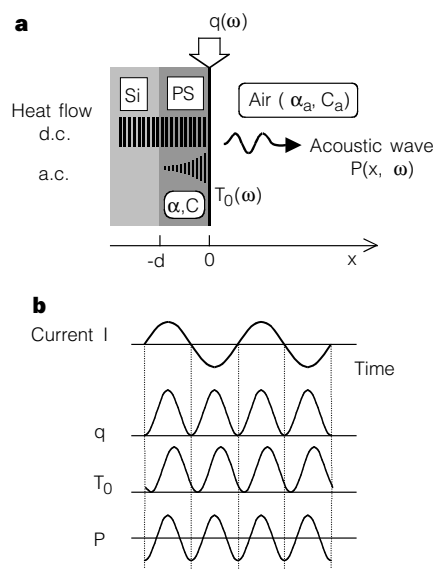


Figure 2 Device operation. **a**, The air/thin-Al-film porous-silicon (PS)/c-Si structure and the coordinate configuration. See text for details. **b**, current I introduced into the top electrode induces the surface temperature change T_0 by Joule's heat q , which produces acoustic pressure P .

Table 1 Thermal properties of Si and SiO₂

	α (W m ⁻¹ K ⁻¹)	C (10 ⁶ J K ⁻¹ m ⁻³)	$\alpha C / (\alpha C)_{\text{c-Si}}$
c-Si	168	1.67	1
Porous silicon	1	0.7	1/400
SiO ₂	1.4	2.27	1/88

Shown are thermal conductivity, α , and heat capacity per unit volume, C , of c-Si in comparison to typical values for porous silicon. Data for SiO₂ are also shown for reference. Note that the relative value of the product αC of porous silicon is extremely low.

where P_A is atmospheric pressure, T_A is room temperature, v is the sound velocity, $\gamma = C_p/C_v = 1.4$, k is the wavenumber of free-space sound, α_a is the thermal conductivity of air, and C_a is the heat capacity per constant unit volume of air. The assumptions in this analysis are as follows: $k \ll \sqrt{\omega C_a / \alpha_a}$ (that is, the sound wavelength is much larger than thermal diffusion length), $\alpha C \gg \gamma \alpha_a C_a$ (that is, the heat flow into the device is much larger than that into the air), and the porous silicon itself does not vibrate. Too large a value of d causes a stationary temperature rise on the surface (which is useless for our purposes) and, in contrast, too small a d decreases the signal of the acoustic pressure amplitude itself. We note that the ratio $|P(\omega)|/|q(\omega)|$ is constant, and independent of ω . This means that an ideal, flat frequency response is expected from this device.

We now evaluate the product αC , because it determines the efficiency of the operation as suggested by equation (3). Table 1 shows typical thermal data of porous silicon in comparison to that of c-Si (refs 12, 13). The data of SiO₂ are also shown for reference. It is clear that the value of αC is $\sim 1/400$ that of c-Si: this big difference in αC between porous silicon and c-Si would prevent heat transfer of an alternating component to the inside of the device, while a possible stationary d.c. component would be quickly removed into the highly thermally-conductive c-Si substrate.

The measured acoustic pressure amplitudes are plotted in Fig. 3 as a function of output frequency for a sinusoidal Joule's heating power of 1 W cm^{-2} . A significantly high acoustic pressure is observed over a wide frequency range up to 100 kHz. The limit of upper measurement frequency at 100 kHz is simply due to the specification of our measurement system; and the condition of equation (1) is satisfied at frequencies above 5 kHz. As predicted by theory, sound intensity is independent of frequency in the high-frequency range where the wavelength is smaller than the device size, although some fluctuations caused by environmental reflection noise are seen. The value of the pressure also coincides with that calculated from equation (2) and the data in Table 1, as P (in Pa) = $0.07 \times q$ (in W cm^{-2}).

To compare (in Pa) the efficiency of our device with that of conventional methods is not straightforward, as the output acoustic pressure of our device is proportional to the input power per unit area, while that of piezoelectric or electrostatic devices is proportional to the input voltage. For conventional devices, an average membrane displacement of 12.6 nm V^{-1} at 1.7 MHz has been reported⁵: this corresponds to 55 Pa V^{-1} , which we take as the highest efficiency at present. A PZT bimorph transducer with impedance matching cone (EFRTUB50K5, Matsushita) generates 2 Pa V^{-1} at 40 kHz when it is measured at distance of 30 cm. In the survey by Manthey *et al.*,¹ typical efficiencies of both piezoelectric and (non-MEMS) electrostatic types are given as $0.1\text{--}1 \text{ Pa V}^{-1}$ at 200 kHz when measured at a distance of 1 m. (Here MEMS indicates micro-electrical-mechanical systems.) As the impedance of our experimental device was 5Ω , a 1 cm^2 device driven by a 5 V source gives a 0.4 Pa plane wave whose intensity is not much less than that of the conventional resonant device, though our device consumes more power. We note that the frequency range of our device is broad, unlike the resonant devices, and that its efficiency increases as the sound intensity increases. The thermo-acoustic coupling factor, which is defined as the square root of the ratio of the acoustic

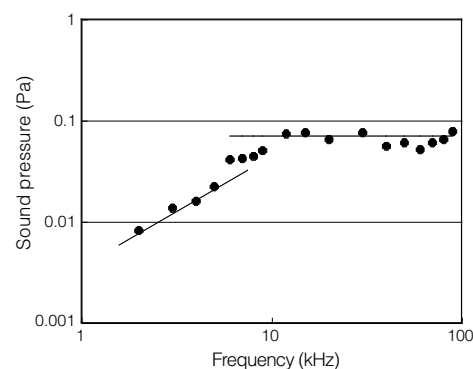


Figure 3 Experimental results. Shown is the measured acoustic pressure amplitude for the experimental device as a function of output frequency. The electrical power input was 1 W cm^{-2} . In the high-frequency range (where the wavelength is smaller than the device size), the sound pressure is independent of frequency, as predicted by theory.

power output to the input electric power, is 0.03% at 1 W cm^{-2} , while that of the conventional method reaches 10% . This low coupling factor is a present drawback of our non-optimized device. But the freedom to select its electrical impedance, and a high intensity free from the upper bound of conventional methods, are also advantages of our device, as are mechanical toughness, ideally flat frequency characteristics, and the potential to assemble a phased array of such devices with low crosstalk.

As a result of the scaling principle in thermal conduction phenomena, the use of dot electrode structures (in which the heat exchange is concentrated in small islands) should greatly improve the efficiency and output acoustic pressure of our devices. Moreover, such construction could prove useful in two-dimensional array fabrication because it provides a large wiring space. On the other hand, as suggested by the data in Table 1, the use of oxidized porous silicon would be a promising and practical approach to enhancing the efficiency.

Although we have not performed rigorous tests of its stability, this device is free from the often-quoted instability of porous silicon. The porous silicon layer is protected by the aluminium electrode from the atmosphere, and is not exposed to an intense electric field. (It is conceivable that oxidation might in fact upgrade the efficiency rather than degrade it.) We can confirm that this device worked well after three months at room temperature, humidity and pressure.

Particular attention has been paid to the visible luminescence of porous silicon^{14–16}. It is closely related to an optical bandgap widening, induced by strong quantum confinement in silicon nanocrystallites with the same band dispersions as c-Si (ref. 17). But complete carrier depletion in nanocrystallites associated with strong confinement, on the other hand, leads to extremely low values of α and C . The ability to control these parameters over a wide range, and the process compatibility with ultra-large-scale-integration (ULSI) technology—including a large difference in the thermal properties between porous silicon and c-Si—suggests applications of porous silicon in acoustic integrated devices. □

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- Manthey, W., Kroemer, N. & Magori, V. Ultrasonic transducers and transducer arrays for applications in air. *Meas. Sci. Technol.* **3**, 249–261 (1992).
- Mo, J. H., Fowlkes, J. B., Robinson, A. L. & Carson, P. L. Crosstalk reduction with a micromachined diaphragm structure for integrated ultrasonic transducer arrays. *IEEE Trans. Ultrasonics Ferroelectr. Freq. Control* **39**, 48–53 (1992).
- Goldberg, R. L. & Smith, S. W. Multilayer piezoelectric ceramics for two-dimensional array transducers. *IEEE Trans. Ultrasonics Ferroelectr. Freq. Control* **39**, 761–771 (1994).
- Soh, H. T., Atalar, A. & Khuri-Yakub, B. T. Surface micromachined capacitive ultrasonic transducers. *IEEE Trans. Ultrasonics Ferroelectr. Freq. Control* **45**, 678–690 (1998).
- Haller, M. I. & Khuri-Yakub, B. T. A surface micromachined electrostatic ultrasonic air transducer. *IEEE Trans. Ultrasonics Ferroelectr. Freq. Control* **43**, 1–6 (1998).
- Arnold, H. D. & Crandall, I. B. The thermophone as a precision source of sound. *Phys. Rev.* **10**, 22–38 (1917).

7. Amato, G., Benedetto, G., Barino, L., Brunetto, N. & Spagnolo, R. Photothermal and photoacoustic characterization of porous silicon. *Opt. Eng.* **36**, 423–431 (1997).
8. Calderon, A., Alvarado-Gil, J. J. & Gurevich, Y. G. Photothermal characterization of electrochemical etching processed n-type porous silicon. *Phys. Rev. Lett.* **79**, 5022–5025 (1997).
9. Cullis, A. G., Canham, L. T. & Calcott, P. D. J. The structural and luminescence properties of porous silicon. *J. Appl. Phys.* **82**, 909–965 (1997).
10. Holman, J. P. *Heat Transfer* (McGraw-Hill, New York, 1963).
11. McDonald, F. A. & Wetsel, G. C. Jr Generalized theory of the photoacoustic effect. *J. Appl. Phys.* **49**, 2313–2322 (1978).
12. Lang, W., Drost, A., Steiner, P. & Sandmaier, H. The thermal conductivity of porous silicon. *Mater. Res. Soc. Symp. Proc.* **358**, 561–566 (1995).
13. Lang, W. in *EMIS Data Review Ser. no. 18, Properties of Porous Silicon* (ed. Canham, L.) 138–141 (IEE, London, 1997).
14. Canham, L. Silicon quantum wire array fabrication by electrochemical and chemical dissolution of wafers. *Appl. Phys. Lett.* **57**, 1046–1048 (1990).
15. Koshida, N. & Koyama, H. Visible electroluminescence from porous silicon. *Appl. Phys. Lett.* **60**, 347–349 (1992).
16. Hirschman, K. D., Tsybeskov, L., Duttgupta, S. P. & Fauchet, P. M. Silicon-based visible light-emitting devices integrated into microelectronic circuits. *Nature* **384**, 338–341 (1996).
17. Suda, Y., Obata, K. & Koshida, N. Band dispersions in photoluminescent porous silicon. *Phys. Rev. Lett.* **80**, 3559–3562 (1998).

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Layered double hydroxides exchanged with tungstate as biomimetic catalysts for mild oxidative bromination

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The manufacture of a range of bulk and fine chemicals, including flame retardants, disinfectants and antibacterial and antiviral drugs, involves bromination¹. Conventional bromination methods typically use elemental bromine, a pollutant and a safety and health hazard. Attempts to develop alternative and more benign strategies have been inspired by haloperoxidase enzymes, which achieve selective halogenation at room temperature and nearly neutral pH by oxidizing inorganic halides with hydrogen peroxide^{2,3}. The enzyme vanadium bromoperoxidase has attracted particular interest^{4,5} in this regard, and several homogeneous inorganic catalysts mimicking its activity are available^{6–11},

although they are limited by the requirement for strongly acidic reaction media. A heterogeneous mimic operating at neutral pH has also been reported¹², but shows only modest catalytic activity. Here we describe a tungstate-exchanged layered double hydroxide that catalyses oxidative bromination and bromide-assisted epoxidation reactions in a selective manner. We find that the catalyst is over 100 times more active than its homogeneous analogue. The low cost and heterogeneous character of this system, together with its ability to operate efficiently under mild conditions using bromides rather than elemental bromine, raise the prospect of being able to develop a clean and efficient industrial route to brominated chemicals and drugs and epoxide intermediates.

Layered double hydroxides (LDH) consist of alternating cationic $M(II)_{1-x}M(III)_x(OH)_2^{x+}$ and anionic $A^{n-} \cdot zH_2O$ layers¹³. The positively charged layers contain edge-shared metal $M(II)$ and $M(III)$ hydroxide octahedra, with charges neutralized by A^{n-} anions located in the interlayer spacing or at edges of the lamellae. Small hexagonal LDH crystals with $M(II)_{1-x}Al_x(OH)_2(Cl)_x \cdot zH_2O$ composition (referred to as (MAL) LDH) were synthesized following existing procedures (here $M(II)$ is Mg ($x = 0.3$) or Ni ($x = 0.28$); the crystals were 50–100 nm in size with external surface areas of $\sim 100 \text{ m}^2 \text{ g}^{-1}$)¹³. Tungstate was exchanged onto chloride-saturated (MgAl) LDH or (NiAl) LDH to an extent of 12%. Chemical analysis showed complete uptake of the metal anion and release of equivalent amounts of chloride ions. X-ray powder diffraction patterns of the initial LDH materials and of the tungstate-exchanged LDH (WO_4^{2-} LDH) hardly differ in the range $2\theta = 3^\circ$ – 65° . Lattice parameters calculated for rhombohedral symmetry $3R$ give c values of 2.346 and 2.376 nm ($3d_{001}$), and a values of 0.303 and 0.306 nm ($2d_{110}$) for (NiAl) LDH and (MgAl) LDH, respectively. The observed basal spacings remain unchanged after the anion exchange, which indicates that WO_4^{2-} should be mainly located in edge positions. Raman spectra of WO_4^{2-} LDH show vibrations (in cm^{-1}) at 345 (ν_2, ν_4), 838 (ν_3) and 931 (ν_1), characteristic for isolated tetrahedral tungstate (T_d)¹⁴. Hence there is no evidence for formation of polymeric tungsten compounds on the surface of the tungstate-exchanged LDHs that we used.

An assay for bromoperoxidase activity is the conversion of phenol red (phenolsulphonphthalein) into bromophenol blue (tetrabromophenolsulphonphthalein)^{4,5}. Conversion of 0.05 mM phenol red (wavelength of maximum absorption $\lambda_{\text{max}} = 429 \text{ nm}$) into bromophenol blue ($\lambda_{\text{max}} = 598 \text{ nm}$) was complete within 32 min in the presence of WO_4^{2-} (MgAl)LDH, 0.1 M NH_4Br and 2.5 mM H_2O_2 . This corresponds to 17 catalytic cycles in tungsten. The production of bromophenol blue presumably proceeds through H_2O_2 coordination on the metal and formation of peroxo-metal species. These were identified in the ultraviolet–visible reflectance spectra (for example, 330 nm for the tetraperoxo tungstate anion). When Br^- is added, the peroxo tungsten species react in

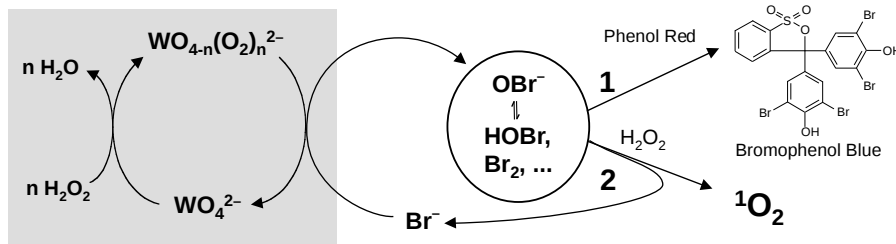


Figure 1 Catalytic cycle in bromination with WO_4^{2-} LDH. H_2O_2 binds to tungstate to form peroxotungstate at the surface of the LDH. Electrostatic attraction brings bromide to the surface, facilitating transfer of the activated oxygen atom from peroxotungstate to Br^- . Reactions of 2-electron-oxidized Br species in solution: electrophilic bromination of phenol red into bromophenol blue (route 1), and

bromide-assisted 1O_2 generation from H_2O_2 (route 2). A preparative experiment of (route 1) was run on a 250-ml scale, followed by work-up via extraction with (n - C_6H_{13}) $_4$ NCl to a CH_2Cl_2 layer. The 1H NMR spectrum of the bromophenol blue product (7.29, s, 4H; 7.01 and 7.90, d, 1H each, $J = 7.5 \text{ Hz}$; 7.41 and 7.50, t, 1H each, $J = 7.5 \text{ Hz}$, TMS standard) is identical to that of authentic bromophenol blue.

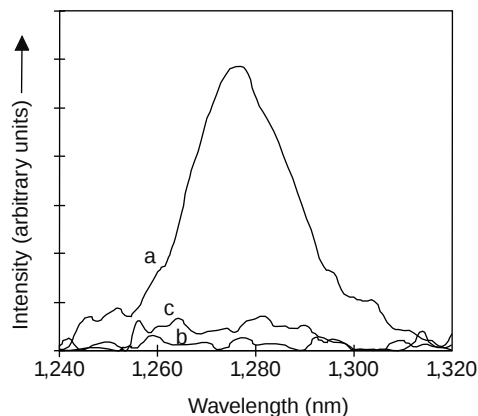


Figure 2 Bromide-assisted $^1\text{O}_2$ generation, catalysed by $\text{WO}_4^{2-}(\text{MgAl})\text{LDH}$. Trace a, near-infrared emission from $^1\text{O}_2(^1\Delta_g)$ in the reaction of 100 mM H_2O_2 and 20 mg $\text{WO}_4^{2-}(\text{MgAl})\text{LDH}$ with 0.1 M NH_4Br in $\text{D}_2\text{O}-\text{CD}_3\text{OD}$ (3 ml total volume). In the absence of Br (trace b), or in the presence of 55 mM monochlorodimedone (trace c), hardly any emission is detected. The excess monochlorodimedone is used to trap any oxidized bromine species, so that Br^+ is not available to form $^1\text{O}_2$.

turn with bromide, eventually reforming tungstate. Subsequently, the oxidized bromine species brominate phenol red (Fig. 1, route 1).

An important diagnostic reaction for the enzyme vanadium bromoperoxidase (VBPO) is the catalytic generation of dioxygen in the absence of an organic substrate¹⁵. This dioxygen originates from a 2e^- oxidation of H_2O_2 by oxidized bromine, and is in the singlet excited state according to spin conservation rules (Fig. 1, route 2)^{16,17}. Singlet molecular oxygen ($^1\text{O}_2$) can be detected by its luminescence at 1,268 nm, due to the forbidden $^1\text{O}_2(^1\Delta_g) \rightarrow ^3\text{O}_2(^3\Sigma_g^-)$ transition¹⁸. Trace a in Fig. 2 shows the strong near-infrared emission when H_2O_2 reacts with $\text{WO}_4^{2-}(\text{MgAl})\text{LDH}$ and NH_4Br in a deuterated solvent, indicative of the liberated oxygen being in the singlet state, and not in the triplet state usually generated by catalase and its mimics.

For optimal regio- and stereoselectivity in bromination of unsaturated compounds, the formation of radicals should be avoided. The presence of radicals can be probed by using 2,3-dimethoxytoluene (DMT) as substrate. With $\text{WO}_4^{2-}(\text{NiAl})\text{LDH}$ (80 mg), 0.33 M NH_4Br , 33 mM DMT and 55 mM H_2O_2 in $\text{MeOH}:\text{H}_2\text{O}$ ring-substituted bromo-DMT is exclusively formed.

The oxidized bromine species is therefore ' Br^+ ' rather than $\text{Br}^\cdot$, as a radical reaction would also yield some dimethoxybenzylbromide. Evidence for the electrophilic nature of the bromination is also found in the relative reactivities of *para* and *meta* substituted styrenes. Such effects are quantified in the Hammett equation: $\log k_r = \rho^{(+)}\sigma^{(+)}$, where the substituent parameter $\sigma^{(+)}$ is positive for electron-withdrawing substituents (and vice versa), k_r is the relative bromination rate of a substituted styrene versus styrene, and $\rho^{(+)}$ is the slope of the plot. Electrophilic reactions are accelerated by electron-donating substituents, and this yields a ρ value <0 , as in the reaction of styrenes with water ($\rho^+ = -3.58$)¹⁹ or with Br_2 in acetic acid ($\rho^+ = -4.8$)²⁰. For bromination of styrenes with $\text{WO}_4^{2-}(\text{MgAl})\text{LDH}$, a plot of relative reaction rates versus σ gives a slope $\rho = -5.5$ ($r^2 = 0.989$; 0.75 mM W on LDH, 33 mM substrate, 0.33 M NH_4Br and 55 mM H_2O_2). Such a firmly negative correlation with the σ parameter proves that the transition state of the bromination is positively charged. Moreover the main products from the styrene reaction are 1-bromo-2-methoxy-2-phenylethane (in MeOH) or 2-bromo-1-phenylethanol (in water); this observation of Markovnikov-directed incorporation of solvent nucleophiles is additional evidence for an electrophilic reaction.

A comparison of catalytic bromination activities of $\text{WO}_4^{2-}\text{LDH}$, dissolved or immobilized commercial bromoperoxidase, and several homogeneous or heterogeneous mimics, reveals three main points (Table 1). First, WO_4^{2-} has a much higher activity in an LDH environment than in solution, even on a total-catalyst-weight basis. For bromophenol blue formation in identical reaction conditions (for example, 2.5 mM H_2O_2), the turnover rate of WO_4^{2-} is enhanced by more than 100 times by immobilization on the positively charged LDH structures (reactions 1–4 to Table 1). This strongly improved activity in mild pH conditions ($6 < -\log[\text{H}^+] < 8$) is in sharp contrast with previous, homogeneous mimics, which generally require strong acid ($-\log[\text{H}^+] < 2.5$) to achieve comparable catalytic activities^{6–11}. We believe that the large positive electric potential of the $\text{WO}_4^{2-}\text{LDH}$ surface induces an enrichment of bromide ions close to the surface. Moreover, the simultaneous presence of bromide and peroxotungstate anions on the catalyst surface decreases the electrostatic repulsion between these negatively charged reaction partners. Overall, spatial organization and electrical shielding are responsible for the superior performance of the $\text{WO}_4^{2-}\text{LDH}$ catalyst. We note that charge shielding also occurs at the active site of the VBPO of *Curvularia inaequalis*²¹, where the negative charge of peroxovanadate is compensated by surrounding Arg, His and Lys residues.

Table 1 Catalytic bromination activities of VBPO mimics

Reaction no.	Catalyst*	[metal] (mM)	$[\text{H}_2\text{O}_2]$ (mM)	TOF $\times 10^4$ (h^{-1})†	Specific activity ($\text{mmol g}^{-1} \text{h}^{-1}$)‡
1	$\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$	0.05	2.5	2,500	0.84
2	$\text{WO}_4^{2-}(\text{MgAl})\text{LDH}\ddagger$	0.05	2.5	330,000	6.2
3	$\text{WO}_4^{2-}(\text{MgAl})\text{LDH}\ddagger$	0.05	5	710,000	13.3
4	$\text{WO}_4^{2-}(\text{NiAl})\text{LDH}\ddagger$	0.05	5	480,000	9
5	Ti-MCM-48	2.5	10	1,600	0.16 (ref. 12)
6	Ti-MCM-41	0.5	2.5	560	0.047
7	TS-1	0.5	2.5	6	0.00025
8	Febpy_2Y	0.05	2.5	13	0.00078
9	Mnbpy_2Y	0.05	2.5	ND	ND
10	VHPO	–	–	–	600§
11	Immobilized HPO	–	–	–	0.15–6

Specific activities were determined at 298 K (at 700 r.p.m.) with 0.05–0.5 mM metal catalyst, 2.5–5 mM H_2O_2 , 0.1 M NH_4Br in 20 ml solvent ($\text{H}_2\text{O}:\text{MeOH}:\text{THF} = 4:3:2$), and 0.05 mM phenol red or 0.5 mM MCD (monochlorodimedone, used in entries 3, 4, 10 and 11). Concentrations were monitored spectrophotometrically at 290 nm for MCD ($\epsilon_{\text{MCD}} \approx 100 \epsilon_{\text{Br-MCD}}$) or at 429 nm (phenol red).

* Catalysts: Ti-MCM-48 (5 wt% Ti) and Ti-MCM-41 (4 wt% Ti), Ti-containing mesoporous silicates with cubic three-dimensional (MCM-48)¹² or hexagonal one-dimensional pore structure (MCM-41)^{22–23}; TS-1, Ti-silicalite-1²⁴; Febpy_2Y and Mnbpy_2Y , *cis*-[Fe(bpy)₂]²⁺ or *cis*-[Mn(bpy)₂]²⁺ encapsulated in zeolite NaY (bpy = bipyridine)²⁵; VHPO, V-bromoperoxidase from *Corallina officinalis* (Sigma).

† TOF (turnover frequency) = mol $\text{Br}^\cdot$ oxidized per mol of metal per h. Specific activity = mole $\text{Br}^\cdot$ oxidized per total weight of catalyst (g) per h. Full trapping of oxidized Br species by MCD or phenol red is assumed (1 or 4 equivalents, respectively).

‡ To reach an anion exchange degree of 12%, 1g Cl-LDH is stirred in 100 ml aqueous 1.87 mM sodium tungstate (298 K, 24 h), then washed and lyophilized to dryness.

§ Commercial lyophilized protein mixture (10% pure) at 298 K and pH 6.5. Activities of non-commercial enzymes may be superior^{3–5}.

|| Enzyme immobilization generally causes at least a 100-fold reduction of the activity per catalyst weight²⁶; lower values have been reported for immobilized haloperoxidases^{28,29}.

ND, not detected.

Table 2 Preparative catalytic bromination with WO_4^{2-} (NiAl)LDH

Reaction no.	Substrate	MeOH/H ₂ O	Product yields (%)
1*		50/50	 95%
2		0/100	
3		95/5	
4†		95/5	
5†		0/100	
6		50/50	

Reactions were conducted at ambient temperature (700 r.p.m.) for 125 min using 0.75 mM WO_4^{2-} on LDH, 33 mM substrate, 0.33 M NH_4Br , 55 mM H_2O_2 (final concentration) in water or methanol/water mixtures. H_2O_2 is added in five portions. Blank reactions in absence of catalyst gave no reaction within 2 h. Blank reactions in absence of bromides resulted in very small amounts of analogous chlorinated products, presumably from oxidation of Cl^- present in the LDH structure. From olefins small amounts of epoxides are obtained (<1%) via direct mono-oxygen transfer from the peroxotungstates^{30,31}.

* 33 mM H_2O_2 , reaction time 75 min.

† Only one enantiomer of each product is shown.

‡ 100% selectivity.

The second point is that WO_4^{2-} (MgAl)LDH is a vastly more active material than any other solid inorganic VHPO mimic (for example, mesoporous Ti catalysts, prepared as described in refs 22 and 23) or any heterogeneous redox catalysts (reactions 5–9 in Table 1)^{24,25}. Last, while the direct use of V bromoperoxidases results in high activities (reaction 10 in Table 1), immobilization of the enzymes is preferred in industrial applications to improve re-usability and stability towards elevated temperatures or high H_2O_2 concentrations. However, this reduces the activity on a weight basis by a factor of at least 100 (ref. 26). Therefore the WO_4^{2-} -LDH mimic presents an attractive alternative to VHPO enzymes.

During the bromination with WO_4^{2-} LDH, less than 0.2 p.p.m. (that is, less than 0.27% of the total amount) of tungsten was found in solution and the use of recycled catalysts resulted in unaltered activity, supporting the oxidative stability of the catalyst. Both the heterogeneity and the constant catalyst activity are essential for practical implementation.

The WO_4^{2-} -LDH catalyst allows the electrophilic bromination of a wide range of nucleophilic substrates (olefins, 1,3-diketones, aromatics) with high selectivity under mild conditions (Table 2). Most of the H_2O_2 is used for bromide oxidation. For instance, with just one mole of H_2O_2 per mole of diketone, the yield of the mono-brominated product from dimedone is 95% (reaction 1 in Table 2). With alkyl substituted olefins, such as 1-methyl-1-cyclohexene our

method provides a large amount of the epoxide (reaction 5 in Table 2); this epoxidation is at least 100 times slower in the absence of Br. Our system allows the rapid transformation of the initially formed bromohydrin intermediates into the corresponding epoxide because it operates under mild pH conditions (initial pH is 6.7–7.2; final pH is 8 measured in the water suspension). Thus, for suitable substrates, our system is an alternative for the halohydrin route in epoxide production. Epoxides are well-known electrophilic 'building blocks' which are easily integrated into synthetic schemes. Instead of the classic two-step halohydrin reaction, the present catalyst allows one-pot conversion to epoxides without bromohydrin isolation. As the catalytic cycle does not require acidity, even acid-labile epoxides can be isolated²⁷. Moreover, Br^- is recycled from the bromohydrin, making water the only by-product. An exemplary application of our method is the selective production of the 6,7-epoxide of linalool (85% yield based on NMR), an important precursor to fragrances. □

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1. Ullmann's Encyclopedia of Industrial Chemistry 6th edn (Electronic release, Wiley-VCH, Weinheim, 1998).
2. Gribble, G. W. Naturally occurring organohalogen compounds. *Acc. Chem. Res.* **31**, 141–152 (1998).
3. Butler, A. & Walker, J. V. Marine haloperoxidases. *Chem. Rev.* **93**, 1937–1944 (1993).
4. Franssen, M. C. R. & van der Plas, H. C. Haloperoxidases: their properties and their use in organic synthesis. *Adv. Appl. Microbiol.* **37**, 41–99 (1992).
5. Butler, A. in *Bioinorganic Catalysis* (ed. Reedijk, J.) Ch. 13 (Dekker, New York, 1993).
6. Conte, V., Di Furia, F. & Moro, S. Mimicking the vanadium bromoperoxidase reactions: mild and selective bromination of arenes and alkenes in a two-phase system. *Tetrahedr. Lett.* **35**, 7429–7432 (1994).
7. Reynolds, M. S., Morandi, S. J., Raebiger, J. W., Melican, S. P. & Smith, S. P. E. Kinetics of bromide oxidation by (oxalato)oxodiperoxomolybdate(VI). *Inorg. Chem.* **33**, 4977–4984 (1994).
8. Espenson, J. H., Pestovsky, O., Huston, P. & Staudt, S. Organometallic catalysis in aqueous solution: oxygen transfer to bromide. *J. Am. Chem. Soc.* **116**, 2869–2877 (1994).
9. Clague, M. J. & Butler, A. On the mechanism of *cis*-dioxovanadium(V)-catalyzed oxidation of bromide by hydrogen peroxide: evidence for a reactive, binuclear vanadium(V) peroxo complex. *J. Am. Chem. Soc.* **117**, 3475–3484 (1995).
10. Meister, G. E. & Butler, A. Molybdenum(VI)- and tungsten(VI)-mediated biomimetic chemistry of vanadium bromoperoxidase. *Inorg. Chem.* **33**, 3269–3275 (1994).
11. Hamstra, B. J., Colpas, G. J. & Pecoraro, V. L. Reactivity of dioxovanadium(V) complexes with hydrogen peroxide: implications for vanadium haloperoxidase. *Inorg. Chem.* **37**, 949–955 (1998).
12. Walker, J. V. *et al.* Peroxidative halogenation catalyzed by transition-metal-ion-grafted mesoporous silicate materials. *J. Am. Chem. Soc.* **119**, 6921–6922 (1997).
13. Trifiro, F. & Vaccari, A. in *Comprehensive Supramolecular Chemistry* Vol. 7, 251 (Pergamon, Elsevier Science, Oxford, 1996).
14. Nakamoto, K. *Infrared and Raman Spectra of Inorganic and Coordination Compounds* (Wiley-Interscience, New York, 1986).
15. Soedjak, H. S. & Butler, A. Mechanism of dioxxygen formation catalyzed by vanadium bromoperoxidase from *Macrocystis pyrifera* and *Fucus distichus*: steady state kinetic analysis and comparison to the mechanism of V-BrPO from *Ascophyllum nodosum*. *Biochem. Biophys. Acta* **1079**, 1–7 (1991).
16. Held, A. M., Halko, D. J. & Hurst, J. K. Mechanisms of chlorine oxidation of hydrogen peroxide. *J. Am. Chem. Soc.* **100**, 5732–5740 (1978).
17. Everett, R. R., Kanofsky, J. R. & Butler, A. Mechanistic investigations of the novel non-heme Vanadium bromoperoxidases. *J. Biol. Chem.* **265**, 4908–4914 (1990).
18. Primer, A. *Single Oxygen* (CRC, Boca Raton, Florida, 1985).
19. Schubert, W. M. & Keeffe, J. R. The acid-catalyzed hydration of styrenes. *J. Am. Chem. Soc.* **94**, 559–566 (1972).
20. Yates, K., McDonald, R. S. & Shapiro, S. A. Kinetics and mechanisms of electrophilic addition. I. A comparison of second- and third-order brominations. *J. Org. Chem.* **38**, 2460–2463 (1973).
21. Messerschmidt, A. & Wever, R. X-ray structure of a vanadium-containing enzyme: Chloroperoxidase from the fungus *Curvularia inaequalis*. *Proc. Natl. Acad. Sci. USA* **93**, 392–396 (1996).
22. Kresge, C. T., Leonowicz, M. E., Roth, W. J., Vartuli, J. C. & Beck, J. S. Ordered mesoporous molecular sieves synthesized by a liquid-crystal template mechanism. *Nature* **359**, 710–712 (1992).
23. Maschmeyer, T., Rey, T., Sankar, F. & Thomas, J. M. Heterogeneous catalysts obtained by grafting metallocene complexes onto mesoporous silica. *Nature* **378**, 159–162 (1996).
24. Huybrechts, D. R. C., De Bruycker, L. & Jacobs, P. A. Oxyfunctionalization of alkanes with hydrogen peroxide on titanium silicalite. *Nature* **345**, 240–242 (1990).
25. Knops-Gerrits, P.-P., De Vos, D., Thibault-Starzyk, F. & Jacobs, P. A. Zeolite-encapsulated Mn(II) complexes as catalysts for selective alkene oxidation. *Nature* **369**, 543–546 (1994).
26. Kadima, T. A. & Pickard, M. A. Immobilization of chloroperoxidase on aminopropyl-glass. *Appl. Environ. Microbiol.* **56**, 3473–3477 (1990).
27. Rouhi, M. New reaction uncouples epoxidation from acidity. *Chem. Eng. News* 7 July, 6–7 (1997).
28. Itoh, N., Cheng, L., Izumi, Y. & Yamada, H. Immobilized bromoperoxidase of *Corallina pilulifera* as a multifunctional halogenating biocatalyst. *J. Biotechnol.* **5**, 29–38 (1987).
29. Aoun, S. & Babouline, M. Regioselective bromohydroxylation of alkenes catalyzed by chloroperoxidase: Advantages of the immobilization on talc. *J. Mol. Catal. B* **4**, 101–109 (1998).
30. Sheldon, R. A. & Kochi, J. K. *Metal-Catalyzed Oxidation of Organic Compounds* (Academic, New York, 1981).
31. Sels, B. F., De Vos, D. E. & Jacobs, P. A. Selective epoxidations involving anionic peroxotungsten compounds generated *in situ* on layered double hydroxides with various polarities. *Tetrahedr. Lett.* **37**, 8557–8560 (1996).

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Competition among marine phytoplankton for different chelated iron species

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Dissolved-iron availability plays a critical role in controlling phytoplankton growth in the oceans^{1,2}. The dissolved iron is overwhelmingly (~99%) bound to organic ligands with a very high affinity for iron³⁻⁵, but the origin, chemical identity and biological availability of this organically complexed Fe is largely unknown⁶. The release into sea water of complexes that strongly chelate iron could result from the inducible iron-uptake systems of prokaryotes (siderophore complexes)⁷⁻⁹ or by processes such as zooplankton-mediated degradation and release of intracellular material (porphyrin complexes). Here we compare the uptake of siderophore- and porphyrin-complexed ⁵⁵Fe by phytoplankton, using both cultured organisms and natural assemblages. Eukaryotic phytoplankton efficiently assimilate porphyrin-complexed iron, but this iron source is relatively unavailable to prokaryotic picoplankton (cyanobacteria). In contrast, iron bound to a variety of siderophores is relatively more available to cyanobacteria than to eukaryotes, suggesting that the two plankton groups exhibit fundamentally different iron-uptake strategies. Prokaryotes utilize iron complexed to either endogenous⁷⁻⁹ or exogenous siderophores⁹, whereas eukaryotes may rely on a ferrireductase system^{10,11} that preferentially accesses iron chelated by tetradentate porphyrins, rather than by hexadentate siderophores. Competition between prokaryotes and eukaryotes for organically-bound iron may therefore depend on the chemical nature of available iron complexes, with consequences for ecological niche separation, plankton community size-structure and carbon export in low-iron waters.

Extracellular chelators are produced by many marine cyanobacteria and heterotrophic bacteria as part of high-affinity Fe uptake systems^{7,9,12}. Several investigators have noted the similarity between Fe-binding conditional stability constants (K_{cond}) of these siderophores and the uncharacterized ligands in sea water (for both, $\log K_{\text{cond}} = 19-23$)^{4,7,8}. The siderophores produced by various marine prokaryotes have diverse molecular structures, including hydroxamates, catecholates^{7,9}, and mixed functional group (β -hydroxy aspartate/catecholate) ligands¹².

Another likely source of strong chelators in the water column is through release of intracellular ligands by zooplankton grazing¹³ or viral lysis¹⁴. Cells contain many organic molecules that can strongly complex iron. We chose tetrapyrroles to represent this type of cell breakdown ligand, since they are among the most abundant strong chelators of Fe in cells. Tetrapyrroles include porphyrins such as those in chlorophylls, cytochromes and haem proteins⁶, and linear tetrapyrroles such as the phycobilin pigments of cyanobacteria. Like siderophores, tetrapyrroles have very high conditional stability constants for Fe(III) binding that resemble those of the seawater ligands (ref. 4, and A.E.W., unpublished results). Faecal pellets of metazoan and protozoan grazers are highly enriched with phaeopigment porphyrins^{15,16}, and release similar strong iron ligands ($\log K_{\text{cond}} = 22$) during decomposition (A.E.W. and D.A.H., unpublished results). Tetrapyrrole ligands appear to be widely distributed in the ocean; recent ¹⁵N nuclear magnetic resonance results suggest that pyrrole-type compounds are ubiquitous components of marine dissolved organic matter¹⁷.

In our experiments, we compare the uptake of ⁵⁵Fe from equimolar concentrations of different radiolabelled iron chelates and from ⁵⁵Fe added in inorganic form (see Methods). We examine the bioavailability of siderophore- and porphyrin-bound ⁵⁵Fe to natural communities of phytoplankton from the oligotrophic Atlantic Ocean, and to cultures of two species of centric diatoms and two species of the unicellular cyanobacterium *Synechococcus* (see Methods). Our models for marine siderophores are the β -hydroxy aspartate/catecholate siderophore alterobactin A and the bis-catecholate siderophore alterobactin B produced by the marine bacterium *Alteromonas luteoviolacea*¹². We use catecholate and hydroxamate siderophores from terrestrial microorganisms as models for marine siderophores with similar iron-binding functional groups⁷⁻⁹, including enterobactin, ferrioxamine B, and ferrichrome. Porphyrin ligands derived from algal pigments and proteins are represented by phaeophytin, phaeophorbide and protoporphyrin IX.

Results of both the culture and the natural assemblage experiments suggest that eukaryotes and prokaryotes specialize in using different organically-complexed Fe sources. At the Gulf Stream and Sargasso stations, the <1.0- μm size class consists almost entirely of *Synechococcus* and heterotrophic bacteria (Methods). At both sites, this prokaryote-dominated small size class can obtain virtually no ⁵⁵Fe from porphyrin complexes (Fig. 1a, b). In contrast, Fe chelated to the catecholate siderophore enterobactin, as well as that added as inorganic species, is readily taken up. Fe bound to the hydroxamate siderophores ferrioxamine and ferrichrome is also available to the prokaryotic size class, although to a lesser degree than enterobactin-bound Fe (Fig. 1a, b).

This same result is observed in experiments using cultures of the Sargasso Sea isolate *Synechococcus* CCMP 1334, where Fe uptake is 12–20 times higher from enterobactin complexes than from the porphyrin complexes. This species also rapidly assimilates Fe bound to the marine siderophores alterobactin A and B, and Fe bound to the two hydroxamate siderophores (Fig. 2a). The coastal Atlantic isolate *Synechococcus* PCC 7002 likewise obtains siderophore-bound Fe much more efficiently than Fe complexed to the porphyrins phaeophorbide and protoporphyrin IX (Fig. 2b). *Synechococcus* uses Fe bound to a variety of exogenous siderophores, but can obtain very little Fe from porphyrin complexes.

In contrast, eukaryotic phytoplankton prefer porphyrin-complexed Fe. The >1.0- μm size class at the two oligotrophic Atlantic stations is dominated by eukaryotic dinoflagellates and small

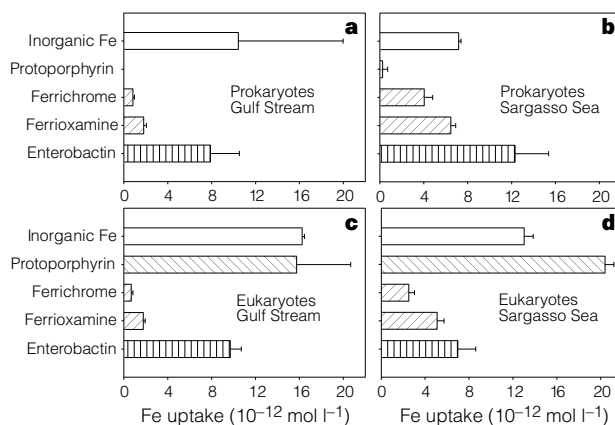


Figure 1 Uptake of organically-bound iron by natural phytoplankton communities. Iron uptake is shown over a 2-d incubation from iron/ligand complexes and from inorganic iron additions. The phytoplankton communities were obtained from the oligotrophic Atlantic; iron was added in inorganic form, and as a porphyrin (protoporphyrin IX), two hydroxamate siderophores (ferrichrome and ferrioxamine B), and a catecholate siderophore (enterobactin). **a, b**, Iron uptake for the prokaryote-dominated 0.2–1.0- μm size class in the Gulf Stream (**a**), and the Sargasso Sea (**b**). **c, d**, Iron uptake for the eukaryote-dominated >1.0- μm size class in the Gulf Stream (**c**) and the Sargasso Sea (**d**).

diatoms (Methods). Although eukaryotes are able to obtain Fe from siderophore complexes, uptake of porphyrin-chelated Fe is 1.6–23 times higher at both stations (Fig. 1c, d). Diatom cultures (*Thalassiosira weissflogii* and *Skeletonema costatum*) also used both siderophore- and porphyrin-bound Fe, but uptake is 1.3–11.5 times higher from the latter sources (Fig. 3). Uptake of Fe by our Fe-replete *T. weissflogii* cultures over the 2-d incubation is similar to steady-state rates previously reported for Fe bound to the synthetic ligands ethylene diamine tetraacetic acid (EDTA) and nitrilotriacetic acid (NTA)¹⁸. Fe uptake rates from siderophore and porphyrin complexes by Fe-limited diatom cultures can be up to an order of magnitude higher, suggesting that the eukaryotic Fe-uptake system is inducible and Fe-regulated (D.A.H., unpublished results).

In all of the experiments, the Fe uptake by phytoplankton from siderophore- and porphyrin-Fe is sometimes equal to (or greater than) the uptake of Fe added in inorganic form (Figs 1, 2, 3). To be consistent with the amounts of ligand-bound Fe added, we use concentrations of inorganic Fe (5 or 10 nM) that exceed the solubility limit of inorganic Fe¹⁹ in sea water. These results support suggestions that organic complexation can increase the solubility of dissolved Fe (ref. 4), thus preventing losses to biologically unavailable oxyhydroxide precipitation²⁰.

Previous work suggests that eukaryotic phytoplankton can access organically-bound Fe through use of a plasma membrane ferrereductase^{10,11}, similar to systems found in some vascular plants²¹ and other eukaryotes²². In this type of non-ligand-specific system, reduction of organically-bound Fe(III) results in dissociation

of the complex, allowing uptake either as inorganic Fe(II) or as Fe(III) after reoxidation. Fe bound by tetradentate, planar porphyrin ligands may be readily available to a non-specific reductase produced by eukaryotes, as it is relatively exposed to the medium and can be accessed from either side of the planar complex. Fe bound by hexadentate siderophore ligands may be less available to a reductase, as this Fe is much more shielded stereochemically by the six surrounding ligating atoms. The use of a non-specific ferrereductase is consistent with our observation that while eukaryotes can obtain some Fe from both porphyrin and siderophore complexes, uptake is greater from porphyrins.

In contrast, prokaryotic phytoplankton are able to utilize Fe chelated by catecholate, hydroxamate or mixed functional group siderophores, but cannot effectively obtain Fe from porphyrin complexes. Uptake of siderophore-bound Fe may occur through recognition of specific functional groups by cell surface siderophore receptors, as *Synechococcus* produces other similar hydroxamate and catecholate ligands⁷. The utilization of exogenous terrestrial and marine siderophores by cyanobacteria confirms earlier suggestions that siderophore 'piracy' may be an important process in competition for Fe in the ocean^{6,9}. As with many terrestrial and enteric prokaryotes²³ and marine heterotrophic bacteria⁹, *Synechococcus* can 'steal' Fe from siderophores produced by other organisms. The lack of substantial Fe uptake from porphyrin complexes suggests that *Synechococcus* does not, however, possess a non-specific ferrereductase system as has been suggested for eukaryotic phytoplankton. Cyanobacteria, with their ability to utilize both inorganic and siderophore-bound iron sources, may have experienced little evolutionary pressure to develop this type of non-selective uptake system.

These observations may shed light on the early evolution of the

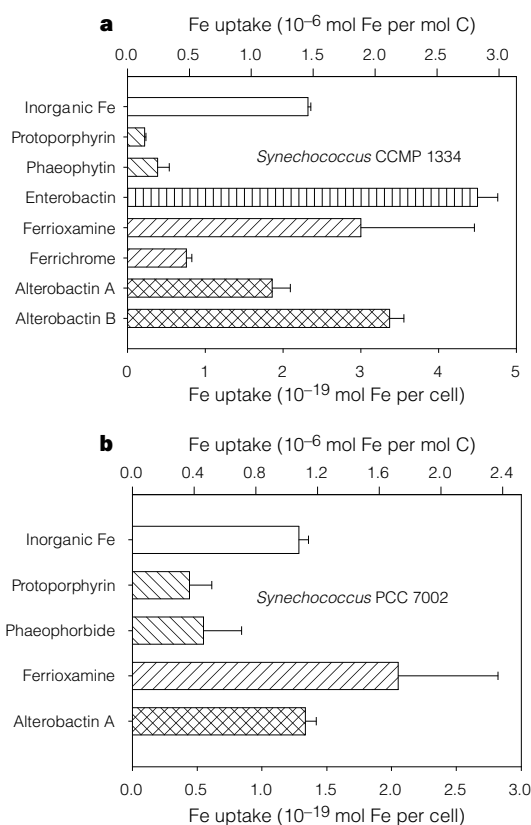


Figure 2 Uptake of organically-bound iron by cyanobacteria cultures. Iron uptake is shown over a 2-d incubation from iron/ligand complexes and from inorganic iron additions. Laboratory cultures of two species of *Synechococcus* were used; iron was added in inorganic form, and as three porphyrins (phaeophytin, protoporphyrin IX, and phaeophorbide), two hydroxamate siderophores (ferrichrome and ferrioxamine B), a catecholate siderophore (enterobactin), and two mixed functional group siderophores (alterobactins A and B). Iron uptake is shown for *Synechococcus* sp. strain CCMP 1334 (**a**), and *Synechococcus* sp. strain PCC 7002 (**b**).

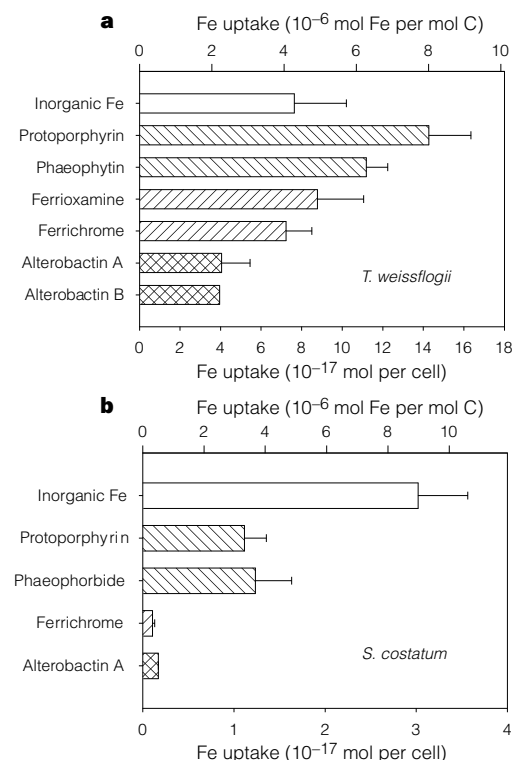


Figure 3 Uptake of organically-bound iron by diatom cultures. Iron uptake is shown over a 2-d incubation from iron/ligand complexes and from inorganic iron additions. Laboratory cultures of two species of diatoms were used; iron was added in inorganic form, and as three porphyrins (phaeophytin, phaeophorbide and protoporphyrin IX), two hydroxamate siderophores (ferrichrome and ferrioxamine B), and two mixed functional group siderophores (alterobactins A and B). Iron uptake is shown for *Thalassiosira weissflogii* strain CCMP 1049 (**a**), and *Skeletonema costatum* strain CCMP 1332 (**b**).

marine Fe biogeochemical cycle. Cyanobacteria first appeared in an anoxic Precambrian ocean where dissolved Fe was readily available as reduced Fe(II). The advent of oxygenic photosynthesis resulted in oxidation to Fe(III), accompanied by a decrease in Fe solubility²⁴ of 6–7 orders of magnitude. Greatly reduced Fe availability under oxic conditions was undoubtedly the driving force behind the development of the many diverse siderophore-mediated Fe-uptake systems at present found in marine prokaryotes. When the first eukaryotic phytoplankton eventually appeared, much of the dissolved Fe in the ocean may have already been complexed by a wide variety of prokaryotic siderophores. Selection under these circumstances would have favoured the development of a non-specific reductase system, capable of allowing eukaryotes to obtain Fe from diverse organic complexes. The appearance of the first eukaryotic protozoan (and later, metazoan) grazers with acidic digestive systems would have provided a new potential organic Fe source: phaeopigment porphyrins and other cell-degradation products. The ferrireductases of eukaryotic phytoplankton, originally developed under selective pressure to obtain Fe from siderophore complexes, may have been uniquely suited to take advantage of this new grazer-produced organic Fe pool.

In the modern ocean, specialization in utilization of different organically-bound Fe sources should help to minimize competition for this limiting resource between the two main taxonomic groups, allowing a degree of niche separation. Much higher surface-to-volume ratios and siderophore production give prokaryotes a large advantage over eukaryotes in obtaining Fe at low concentrations⁶. Nevertheless, eukaryotic phytoplankton (such as diatoms) always make up a variable but significant fraction of community biomass in Fe-limited regimes such as the equatorial Pacific Ocean⁶. The persistence of eukaryotic phytoplankton in low-Fe regions may be possible not only through evolution of lower iron requirements than related coastal species^{24,25}, but also because they can utilize Fe sources such as tetrapyrrole complexes that are not available to cyanobacteria.

The relative importance of siderophores and cell-breakdown products as iron sources may vary from place to place in the euphotic zone. Fe could be complexed mainly by siderophores in prokaryote-dominated oceanic environments (such as the Sargasso Sea or equatorial Pacific), while porphyrin complexation may predominate during heavily grazed coastal diatom blooms. Our results suggest that the chemical nature of organically complexed Fe will in turn influence the outcome of competition for Fe between picoplanktonic cyanobacteria and large eukaryotes such as diatoms. Thus in Fe-limited regimes changes in predominant complexed Fe sources may drive changes in community cell-size structure, a prime determinant of carbon export flux from ocean surface waters²⁶. The preferential use of siderophore-bound Fe by cyanobacteria and tetrapyrrole-bound Fe by eukaryotic autotrophs may be a fundamental feature of marine biogeochemical cycles. □

Methods

Iron/ligand complex preparation. Ligands used in the phytoplankton culture and natural assemblage experiments were de-ferrated²⁷, and residual Fe concentrations were determined⁵. ⁵⁵Fe in 0.01 N HCl was added to each ligand at a 1:5 or 1:10 molar ratio in ligand-free ultraviolet-oxidized sea water (UVSW), that had been passed through a chelex ion exchange resin to remove trace metals, and allowed to equilibrate for 24 h in the dark at 20 °C. Culture uptake media contained 5 nM ⁵⁵Fe chelated to 25 nM ligand (*S. costatum*, *Synechococcus* PCC 7002) or 10 nM ⁵⁵Fe chelated to 50 nM ligand (*T. weissflogii*, *Synechococcus* CCMP 1334), yielding total additions of 0.6×10^6 or 1.2×10^6 Bq ⁵⁵Fe, respectively. Natural assemblage experiments used 5 nM ⁵⁵Fe bound to 50 nM ligand (23.75×10^6 Bq). An inorganic Fe treatment containing the same concentrations of uncomplexed ⁵⁵Fe (in 0.01 N HCl) was included in each experiment. Total dissolved Fe concentrations were measured using competitive ligand equilibration cathodic stripping voltammetry on U.V.-oxidized samples⁵. Molar Fe uptake was calculated from the ⁵⁵Fe activity added and the total dissolved Fe concentration, including the radio-

isotope and Fe remaining in the chelexed UVSW or carried over with the added inoculum (culture experiments, ~0.1 nM and <2.0 nM, respectively), or ambient dissolved Fe (natural assemblage experiments, <0.3 nM). In all experiments, biological uptake used 0 to ~5% of the original total Fe over the course of the incubations.

Natural assemblage experiments. Two experiments used natural plankton assemblages from oligotrophic Atlantic waters at the Bermuda Atlantic Time Series station (31° 40' N, 64° 10' W, 11 May 1998) in the Sargasso Sea, and at a Gulf Stream station (36° 35' N, 70° 58' W, 21 May 1998). Inorganic Fe or ⁵⁵Fe/ligand complexes (ferrioxamine B, ferrichrome, enterobactin, and protoporphyrin IX) were added to replicate 2.7-l acid-washed polycarbonate bottles. Bottles were filled with sea water from a depth of 10 m using a trace-metal-clean pumping system, and incubated in a spectrally corrected deckboard incubator at 40% of ambient light intensity²⁸ for 2 d. The plankton in the samples were then filtered onto 0.2- and 1.0-μm filters, washed with Ti-EDTA-citrate (Ti reagent) to remove extracellular ⁵⁵Fe (ref. 29), and then counted for ⁵⁵Fe radioactivity by liquid scintillation counting. Counting errors were <5% on all samples. Fe uptake by the 0.2–1.0-μm and >1.0-μm size classes was calculated as previously described³⁰ and is reported as mol Fe l⁻¹ obtained over the incubation period. Values and errors are the means and ranges of duplicate bottles for each treatment.

Microscopic examination and measurements of glutaraldehyde-preserved and acridine-orange-stained samples using epifluorescence microscopy and an ocular micrometer²⁸ showed that the composition of the plankton community was similar at both stations. The small size class (0.2–1.0 μm) consisted entirely of prokaryotic taxa, including *Synechococcus* and heterotrophic bacteria. The large size class (>1.0-μm) was dominated by eukaryotic phytoplankton, including dinoflagellates (*Prorocentrum* spp.) and small diatoms (centrics and pennates, 3.0–5.0 μm). Chlorophyll *a* concentrations²⁸ were 0.11 μg l⁻¹ (0.2–1.0 μm) and 0.03 μg l⁻¹ (>1.0 μm) at the Sargasso station, and 0.27 μg l⁻¹ (0.2–1.0 μm) and 0.07 μg l⁻¹ (>1.0 μm) at the Gulf Stream station.

Culture experiments. Experiments used exponential growth phase cultures of the centric diatoms *T. weissflogii* (CCMP 1049) and *S. costatum* (CCMP 1332) and two species of the unicellular cyanobacterium *Synechococcus* sp. (CCMP 1334, a phycoerythrin-containing oceanic isolate, and PCC 7002, a phycocyanin-containing coastal isolate) grown as previously described¹³. Ligands used were the same as those employed in the field experiments, with the addition of phaeophytin, phaeophorbide and alterobactins A and B in some experiments. Uptake from enterobactin complexes was measured for *Synechococcus* CCMP 1334 only. All cultures were grown in a microwave-sterilized medium using freshly obtained axenic inoculum and were not contaminated with bacteria. Cells were filtered and Ti-washed followed by a rinse with 1 l UVSW (diatoms), or centrifuged and rinsed 3 times with chelexed UVSW (cyanobacteria), and then resuspended in 60 ml UVSW in acid-washed polycarbonate bottles with chelexed nutrients (no trace metals) for the uptake portion of the experiment. Initial and final cell densities (cells ml⁻¹) were 1.6×10^4 and 8.8×10^4 (*T. weissflogii*), 1.2×10^4 and 9.2×10^4 (*S. costatum*), 1.7×10^7 and 3.5×10^7 (*Synechococcus* CCMP 1334) and 1.6×10^7 and 2.5×10^7 (*Synechococcus* PCC 7002); pH increased in the uptake bottles from ~8.2 to 8.8–9.0 over the 2 d incubations. Fe uptake was normalized to cell number and to cell C content as determined in separate ¹⁴C-labelled cultures²⁵. Experiments using *S. costatum* and *Synechococcus* PCC 7002 included glutaraldehyde-killed controls, demonstrating that Fe uptake by dead cells was 2–7% of that in live cultures (data not shown). Data are presented as the means and standard deviations of triplicate bottles.

Uptake bottles were incubated for 2 d under continuous cool-white fluorescent illumination (100 μE m⁻² s⁻¹) at 16 °C (diatoms) or 24 °C (cyanobacteria). At the end of the uptake period, 1-ml aliquots were removed from each bottle and preserved with 1% glutaraldehyde for microscopic cell counts. Cultures were then filtered onto 1- or 3-μm (diatoms) or 0.2-μm (cyanobacteria) polycarbonate filters, rinsed with Ti reagent and counted using liquid scintillation counting as described above.

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- Coale, K. H. et al. A massive phytoplankton bloom induced by an ecosystem-scale iron fertilization experiment in the equatorial Pacific Ocean. *Nature* **383**, 495–501 (1996).
- Hutchins, D. A. & Bruland, K. W. Iron-limited diatom growth and Si:N uptake ratios in a coastal upwelling regime. *Nature* **393**, 561–564 (1998).
- Wu, J. & Luther, G. W. Complexation of Fe(III) by natural organic ligands in the Northwest Atlantic Ocean by a competitive ligand method and a kinetic approach. *Mar. Chem.* **50**, 159–177 (1995).

4. Rue, E. L. & Bruland, K. W. Complexation of iron(III) by natural organic ligands in the Central North Pacific as determined by a new competitive ligand equilibration/adsorptive cathodic stripping voltammetric method. *Mar. Chem.* **50**, 117–138 (1995).
5. Witter, A. E. & Luther, G. W. Variation in Fe-organic complexation with depth in the Northwestern Atlantic Ocean as determined using a kinetic approach. *Mar. Chem.* **62**, 241–258 (1998).
6. Hutchins, D. A. in *Progress in Physiological Research* Vol. 11 (eds Chapman, D. & Round, F.) 1–49 (Biopress, Bristol, 1995).
7. Wilhelm, S. W. Ecology of iron-limited cyanobacteria: a review of physiological responses and implications for aquatic systems. *Aquat. Microbial Ecol.* **9**, 295–303 (1995).
8. Butler, A. Acquisition and utilization of transition metal ions by marine organisms. *Science* **281**, 207–210 (1998).
9. Granger, J. & Price, N. M. The importance of siderophores in iron nutrition of heterotrophic marine bacteria. *Limnol. Oceanogr.* **44**, 541–555 (1999).
10. Jones, G. J., Palenik, B. P. & Morel, F. M. M. Trace metal reduction by phytoplankton: the role of plasmalemma redox enzymes. *J. Phycol.* **23**, 237–244 (1987).
11. Weger, H. G. Ferric and cupric reductase activities in the green alga *Chlamydomonas reinhardtii*: experiments using iron-limited chemostats. *Planta* **207**, 377–384 (1999).
12. Reid, R. T., Live, D. H., Faulkner, D. J. & Butler, A. A siderophore from a marine bacterium with an exceptional ferric ion affinity constant. *Nature* **366**, 455–458 (1993).
13. Hutchins, D. A. & Bruland, K. W. Grazer-mediated regeneration and assimilation of Fe, Zn and Mn from planktonic prey. *Mar. Ecol. Prog. Ser.* **110**, 259–269 (1994).
14. Gobler, C. J. *et al.* Elemental release and bioavailability following viral lysis of a marine chrysophyte. *Limnol. Oceanogr.* **42**, 1492–1504 (1997).
15. Strom, S. L. Production of phaeopigments by marine protozoa: results of laboratory experiments analyzed by HPLC. *Deep Sea Res.* **40**, 57–80 (1993).
16. Head, E. J. H. & Harris, L. R. Feeding selectivity by copepods grazing on natural mixtures of phytoplankton determined by HPLC analysis of pigments. *Mar. Ecol. Prog. Ser.* **110**, 75–83 (1994).
17. McCarthy, M., Pratum, T., Hedges, J. & Benner, R. Chemical composition of dissolved organic nitrogen in the sea. *Nature* **390**, 150–154 (1997).
18. Hudson, R. J. M. & Morel, F. M. M. Iron transport in marine phytoplankton: Kinetics of cellular and medium coordination reactions. *Limnol. Oceanogr.* **35**, 1002–1020 (1990).
19. Bruland, K. W., Donat, J. R. & Hutchins, D. A. Interactive influences of bioactive trace metals on biological production in oceanic waters. *Limnol. Oceanogr.* **36**, 1555–1577 (1991).
20. Rich, H. R. & Morel, F. M. M. Availability of well-defined iron colloids to the marine diatom *Thalassiosira weissflogii*. *Limnol. Oceanogr.* **35**, 1555–1577 (1991).
21. Robinson, N. J., Procter, C. M., Connolly, E. L. & Guerinot, M. L. A ferric-chelate reductase for iron uptake from soils. *Nature* **397**, 694–697 (1999).
22. Eide, D. Molecular biology of iron and zinc uptake in eukaryotes. *Curr. Opin. Cell Biol.* **9**, 573–577 (1997).
23. Weinberg, E. D. Cellular regulation of iron assimilation. *Q. Rev. Biol.* **64**, 261–290 (1989).
24. Brand, L. E. Minimum iron requirements of marine phytoplankton and the implications for the biogeochemical control of new production. *Limnol. Oceanogr.* **36**, 1756–1771 (1991).
25. Sunda, W. G. & Huntsman, S. A. Iron uptake and growth limitation in oceanic and coastal phytoplankton. *Mar. Chem.* **50**, 189–206 (1995).
26. Boyd, P. W. & Newton, P. P. Does planktonic community structure determine downward particulate organic carbon flux in different oceanic provinces? *Deep-Sea Res.* **46**, 63–91 (1999).
27. Lewis, B. L. *et al.* Voltammetric estimation of iron(III) thermodynamic stability constants for catecholate siderophores isolated from marine bacteria and cyanobacteria. *Mar. Chem.* **50**, 179–188 (1995).
28. Hutchins, D. A., DiTullio, G. R., Zhang, Y. & Bruland, K. W. An iron limitation mosaic in the California upwelling regime. *Limnol. Oceanogr.* **43**, 1037–1054 (1998).
29. Hudson, R. J. M. & Morel, F. M. M. Distinguishing between extra- and intracellular iron in marine phytoplankton. *Limnol. Oceanogr.* **34**, 1113–1120 (1989).
30. Schmidt, M. A. & Hutchins, D. A. Size-fractionated biological iron and carbon uptake along a coastal to offshore transect in the NE Pacific. *Deep-Sea Res.* **46**, (1999).

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Tracking the evolution of insecticide resistance in the mosquito *Culex pipiens*

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The evolution of pesticide resistance provides some of the most striking examples of darwinian evolution occurring over a human life span. Identification of resistance alleles opens an outstanding framework in which to study the evolution of adaptive mutations from the beginning of pesticide application^{1–3}, the evolution of interactions between alleles (dominance⁴) or between loci (epistasis^{5,6}). Here we show that resistance alleles can also be used as markers to dissect population processes at a microevolu-

tionary scale. We have focused on the antagonistic roles of selection and migration involved in the dynamics of local adaptation with reference to allelic frequencies at two resistance loci in the mosquito *Culex pipiens*. We find that their frequencies follow an annual cycle of large amplitude (25%), and we precisely unravel the seasonal variation of migration and selection underlying this cycle. Our results provide a firm basis on which to devise an insecticide treatment strategy that will better control the evolution of resistance genes and the growth of mosquito populations.

Geographic gradients in allele frequency ('clines') in natural populations can be analysed using migration–selection models^{7,8} to obtain estimates of migration rates and selection coefficients^{9–13}. This powerful method works well for populations near equilibrium, such as hybrid zones¹⁴ that are considered to be “windows on evolutionary processes”¹⁵. The same method (which assumes equilibrium) can be applied to clines shaped by a balance between natural selection and gene flow across a variable environment^{7,8,16,17}, although such local adaptation may be much less stable over time (for example, see ref. 18). This approach has been used to analyse the cases of insect melanism^{19,20} and heavy-metal tolerance in plants²¹. We have used this framework to analyse insecticide resistance but have developed a method that does not require *ad hoc* hypotheses such as neutrality and genetic equilibrium and allows us to study the dynamics through time.

We sampled 8,600 *C. pipiens* mosquitoes during the breeding season (April to October) and the overwintering period (November to March) (Table 1). Sampling was done on ten dates from July 1995 to March 1997 along a north–south transect across organophosphate treated and non-treated areas in Southern France (Fig. 1). In *C. pipiens*, two major loci confer organophosphate resistance. Resistance alleles at the *Ace-1* locus code for a modified acetylcholinesterase (the organophosphate target), whereas those at the *Ester* locus code for overproduced detoxifying esterases^{22–24}. Alleles at these two loci were identified for each individual using a biochemical test and starch-gel electrophoresis, respectively^{22–24}.

We analysed fitness differences within and between loci, ignoring small differences between resistance alleles at the same locus which

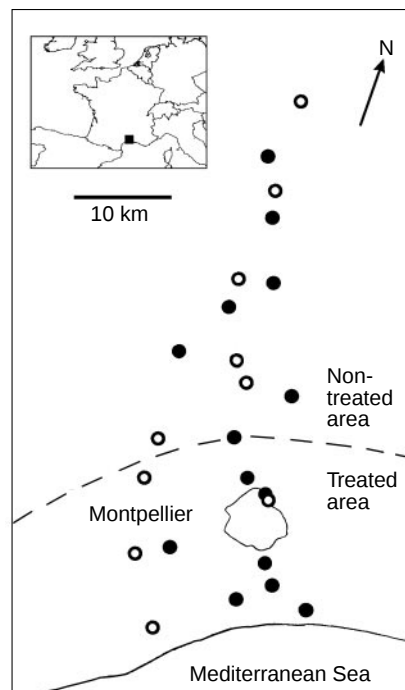


Figure 1 Sample site locations in the north–south transect in Southern France. Diapausing females were sampled in overwintering sites (caves; circles) and larvae or pupae were sampled in larval breeding sites (pools; filled circles). The dashed line indicates the boundary between the treated and non-treated areas.

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Table 1 Sampling dates, number of sampled sites and sample sizes

Models		Descriptive				Migration-selection	
Sample set		Pop	N	Deviance	%TD	Deviance	%TD
Oct. 1997	Breeding sites	8	1,056	52.2 (12)	0.86*	54.2	0.84
Nov. 1995	Overw. sites	10	1,270	14.9 (14)	0.68*	28.4	0.40
Nov. 1996	Overw. sites	10	470	9.4 (14)	0.89*	30.8	0.72
Feb. 1996	Overw. sites	17	1,524	23.1 (21)	0.82*	65.3	0.51
March 1997	Overw. sites	7	308	14.9 (11)	0.71*	34.8	0.33
April 1996	Breeding sites	7	421	25.1 (11)	0.60*	39.3	0.38
May 1996	Breeding sites	6	671	48.1 (10)	0.80*	60.4	0.75
June 1996	Breeding sites	5	500	5.4 (2)	0.94*	7.4	0.85
July 1995	Breeding sites	10	1,734	41.7 (14)	0.94*	52.3	0.92
Aug. 1996	Breeding sites	7	640	20.1 (11)	0.89*	23.2	0.87
All samples		87	8,594	254.7 (120)	0.88*	396.1 (15)	0.82

Pop, number of sampled sites; N, sample size. The residual deviance, the number of degrees of freedom used (in parentheses), and the explained part of the total deviance (%TD) are shown for both the cline descriptive analysis and the migration-selection model.

* A significant decrease of resistance alleles from the coast was detected using descriptive models ($P < 0.001$).

have been estimated separately in longer-term studies^{23,25}. Individuals were pooled into classes {E}/[O] and {R}/[S] for the presence/absence of at least one resistance allele at the *Ester* (E) and the *Ace.1* (R) locus, respectively. The phenotypes were therefore treated as being {S,O}, {S,E}, {R,O} or {R,E}.

For each sampling date, we found a significant decrease of {R} and {E} frequency with distance from the coast (Table 1) using a descriptive model (see Methods). We also found that the cline shape followed an annual cycle: cline shape differed across seasons and was almost identical within seasons from one year to the next (Fig. 2). These clines can be interpreted as the consequence of local adaptation in a finite environment where migration and selection act as antagonistic forces¹⁷. A one-dimensional cline can be maintained at an equilibrium across a finite treated and non-treated area when resistance alleles confer a constant relative fitness advantage in the presence of insecticide but a relative fitness disadvantage (referred to as the 'cost' of resistance) in its absence². Also, it is required that the intensity of selection and the size of the treated area are large enough relative to the intensity of gene flow^{17,24}. For more than one locus, each cline is modified by the indirect selection due to linkage disequilibria and these conditions are more easily met²⁶.

Our data provide a rare opportunity to go beyond these snapshot pictures and to estimate migration and selection coefficients as well as their variation across time. We analysed the annual cycle of the clines as a consequence of annual variation in insecticide applications and alternations between breeding and overwintering seasons. We performed a maximum likelihood analysis (see Methods) using deterministic equations describing migration and selection to infer the allelic distribution at each step of the cycle and assuming the whole cycle is stable. One-dimensional clines were simulated using a series of demes connected by migration (see Methods). Some of the parameters required, such as the recombination rate (14.5%) between the *Ace.1* and *Ester* loci, and the size of the treated area ($L = 20$ km) were estimated from external data²⁴. Frequencies were computed separately for each sex during overwintering as only females overwinter as adults, whereas males survive as spermatozoa in fertilized females. The cycle was divided into nine intervals (between each sampling date: Fig. 3), within which selection and migration parameters are estimated, $p_i = (s_a, s_e, c_a, c_e, \Delta)$. Parameters s and c correspond to the selection coefficients due to insecticide treatments and the cost of resistance, respectively. The subscripts refer to loci (a for *Ace.1* and e for *Ester*); Δ is the migration distribution characterized by a mean (μ) and a variance (σ^2).

The best-fitting parameters explained 82% of the total deviance (the deviance is -2 times the log-likelihood) over the annual cycle, and the fit of each cline was close to the goodness of fit from purely descriptive models (88%), although 8 times more parsimonious (only migration and selection parameters are estimated; see Table 1). According to these results, the cycle can be divided into five stages

(Fig. 3). During stage I (when insecticides are applied), selection coefficients due to insecticide treatments are positive (for example, $s_a = 0.33$, $s_e = 0.19$ in summer), causing the frequencies of resistance alleles to increase in the treated area. The clines become steeper owing to the antagonistic effect of selection due to insecticide treatments and the cost of resistance (for example, $c_a = 0.11$ and $c_e = 0.07$ in summer) despite the homogenizing effect of strong migration ($\sigma = 6.6$ km generation^{-1/2}). At the end of this stage, migration-selection equilibrium is not completely reached (estimates of selection assuming the cline of July 1995 was at equilibrium²⁴ were therefore underestimated for both loci, about 0.01 for fitness cost and 0.03 for insecticide selection). Stage II corresponds to the end of the breeding season in the absence of insecticide ($s_a = s_e = 0$); the clines start to decay as a result of fitness costs ($c_a = 0.11$ and $c_e = 0.05$) and gene flow ($\sigma = 6.6$ km generation^{-1/2}). Stage III corresponds to an intense ($\sigma = 14.6$ km generation^{-1/2}) and directional ($\mu = 12.4$ km towards North) migration to the overwintering sites which strongly homogenizes frequencies and creates high positive linkage disequilibria (3–5%) along the transect, as expected. This asymmetrical migration explains the surprising increase in resistance alleles in the non-treated area for both loci in both November 1995 and 1996 (see also ref. 27). During the overwintering period (stage IV), resistance

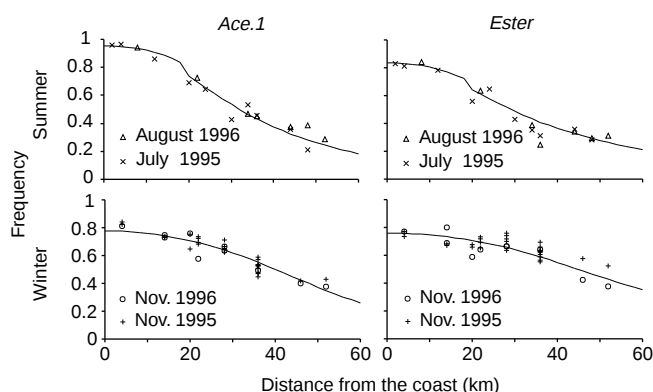


Figure 2 Summer and winter clines of 1995 and 1996. {R} frequency for *Ace.1* locus and {E} frequency for *Ester* locus are indicated as functions of distance from the coast in kilometres. Symbols represent observed frequencies and lines represent the clines fitted using the migration-selection model (see text). During the treatment periods in summer, resistance alleles are selected for in the treated area, producing the steep clines and high frequency of resistance alleles. Owing to the cost of resistance and gene flow, this frequency decreases when the treatments are interrupted, leading to shallow clines and lower frequencies of resistance alleles in winter. The occurrence and stability of this annual cycle are strong evidence that insecticide treatments and the cost of resistance act as antagonistic selective pressures.

alleles decrease in frequency owing to severe fitness costs ($c_a = 0.51$, $c_e = 0.26$) in the absence of movement ($\mu = \sigma = 0$) of diapausing females. Previous estimates of costs associated with *Ace.1* from local empirical data were similarly high ($c_a = 0.63$)^{22,27}. The last stage (V) corresponds to the first spring generation where resistance alleles increase even in the absence of insecticides. This increase is due to winter fitness costs on resistance alleles being higher in females than in spermatozoa (only an indirect selection operates on spermatozoa through the correlation between genotypes of assorted males and females). The net effect of natural selection over the whole winter is therefore expected to be approximately half of that estimated on females, which agrees well with our estimates (c_a and c_e estimated from the beginning of overwintering to the first spring generation were found to be 52 and 51%, respectively, of those estimated over the overwintering period).

A precise understanding of the conditions under which insecticide resistance occurs is necessary for the development of successful management strategies²⁸. Precise estimates of the migration distribution and costs of resistance allow us to compute a critical area of insecticide application below which resistance alleles fall to extinction²⁸. Using our results, this area is about half the actual area of treatment for resistance alleles of organophosphate insecticides. Unrelated insecticides such as *Bacillus sphaericus* could then

be used on the remaining part to achieve the same treated area size but without the evolution of organophosphate resistance. □

Methods

Descriptive cline models. The frequency cline of resistance alleles at locus i and time j was fitted according to a scaled negative exponential^{23,25}, $h_{ij} \exp(-a_{ij}x^2)$, where x is the distance from the coast, h_{ij} the maximum frequency of resistance alleles at locus i and time j , and a_{ij} is the rate of decrease of resistance alleles. Distributions of phenotypes were computed using allelic distributions²⁵, assuming that each locus was at Hardy–Weinberg equilibrium and allowing for linkage disequilibria in each population. The likelihood of a sample was computed from the phenotypic multinomial distribution and maximized for parameter estimation.

Migration–selection simulations. One-dimensional clines were simulated on a lattice. We used a binomial migration distribution reflected at one edge of the lattice to take into account the presence of the sea^{25,27}. Denoting the number (0, 1 or 2) of resistance alleles as A and E for the *Ace.1* and *Ester* loci, respectively, the fitness of an individual was computed as

$$1 - g(x) \left[\frac{s_a}{2} (2 - A) + \frac{s_e}{2} (2 - E) \right] - \frac{c_a A}{2} - \frac{c_e E}{2},$$

where $g(x) = 1$ for $x < 20$ km (treated zone) and zero otherwise. Co-dominance was assumed for insecticide resistance and for the cost of resistance, and epistasis between loci was neglected⁶. The order of the life cycle was assumed to be reproduction–migration–selection. Algorithms of migration and selection were checked using analytical results for one locus¹⁷. The number of generations corresponding to each stage of the cycle is indicated in Fig. 2 and is based on field estimations of generation time (B.D., G.T., C. Chevillon and R.M., manuscript in preparation).

Maximum likelihood estimation. Let n_{ijk} and g_{ijk} be the number and expected frequency of individuals having phenotype i in population j at time k . Then, the log-likelihood of observing all the data is

$$\sum_i \sum_j \sum_k n_{ijk} \times \ln(g_{ijk}),$$

where the expected frequencies of phenotypes (g_{ijk}) were computed using a large enough number of iterations of the annual cycle to ensure its stability. Maximum likelihood estimates of parameters were computed simultaneously using the Metropolis algorithm adapted from Barton¹⁰. Model selection was performed using the Akaike information criterion corrected for overdispersion (QAIC = scaled deviance + $2 \times$ degrees of freedom)²⁹. The scaling factor is the ratio of residual deviance over residual degrees of freedom.

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- Mallet, J. The evolution of insecticide resistance: have the insects won? *Trends Ecol. Evol.* **4**, 336–340 (1989).
- McKenzie, J. A. *Ecological and Evolutionary Aspects of Insecticide Resistance* (Landes, Austin, 1996).
- Mutero, A., Pralavorio, M., Bride, J. M. & Fournier, D. Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase. *Proc. Natl Acad. Sci. USA* **91**, 5922–5926 (1994).
- Bourguet, D., Lenormand, T., Guillemaud, T., Marcel, V. & Raymond, M. Variation of dominance of newly arisen adaptive genes. *Genetics* **147**, 1225–1234 (1997).
- Davies, A. G. *et al.* *Scalloped wings* is the *Lucilia cuprina* *Notch* homologue and a candidate for the Modifier of fitness and asymmetry of diazinon resistance. *Genetics* **143**, 1321–1337 (1996).
- Raymond, M., Heckel, D. & Scott, J. G. Interaction between pesticide genes: model and experiment. *Genetics* **123**, 543–551 (1989).
- Haldane, J. B. S. The theory of a cline. *J. Genet.* **48**, 277–284 (1948).
- May, R. M., Ender, J. A. & McMurtrie, R. E. Gene frequency clines in the presence of selection opposed by gene flow. *Am. Nat.* **109**, 659–676 (1975).
- Barton, N. H. The structure of the hybrid zone in *Uroderma bilobatum* (Chiroptera: Phyllostomidae). *Evolution* **36**, 863–866 (1982).
- Szymura, J. M. & Barton, N. H. Genetic analysis of a hybrid zone between the fire-bellied toads, *Bombina orientalis* and *Bombina variegata*, near Cracow in southern Poland. *Evolution* **40**, 1141–1159 (1986).
- Mallet, J. *et al.* Estimates of selection and gene flow from measure of clines width and linkage disequilibrium in *Heliconius* hybrid zones. *Genetics* **124**, 921–936 (1990).
- Sites, J. W., Barton, N. H. & Reed, K. M. The genetic structure of a hybrid zone between two chromosome races of the *Sceloporus grammicus* complex (Sauria, Phrynosomatidae) in central Mexico. *Evolution* **49**, 9–36 (1995).
- Porter, A. H., Wenger, R., Geiger, H., Scholl, A. & Shapiro, A. M. The *Pottia daphidice-edusa* hybrid zone in northwestern Italy. *Evolution* **51**, 1561–1573 (1997).
- Barton, N. H. & Hewitt, G. M. Adaptation, speciation and hybrid zones. *Nature* **341**, 497–503 (1989).
- Harrison, R. G. in *Oxford Surveys in Evolutionary Biology* (eds Antonovics, J. & Futuyma, D.) 69–128 (Oxford University Press, Oxford, 1990).
- Slatkin, M. Gene flow and selection in a cline. *Genetics* **75**, 733–756 (1973).
- Naglaki, T. Conditions for the existence of clines. *Genetics* **80**, 595–615 (1975).
- Cook, L. M., Dennis, R. L. H. & Mani, G. S. Melanic morph frequency in the peppered moth in the Manchester area. *Proc. R. Soc. Lond. B* **266**, 293–297 (1999).

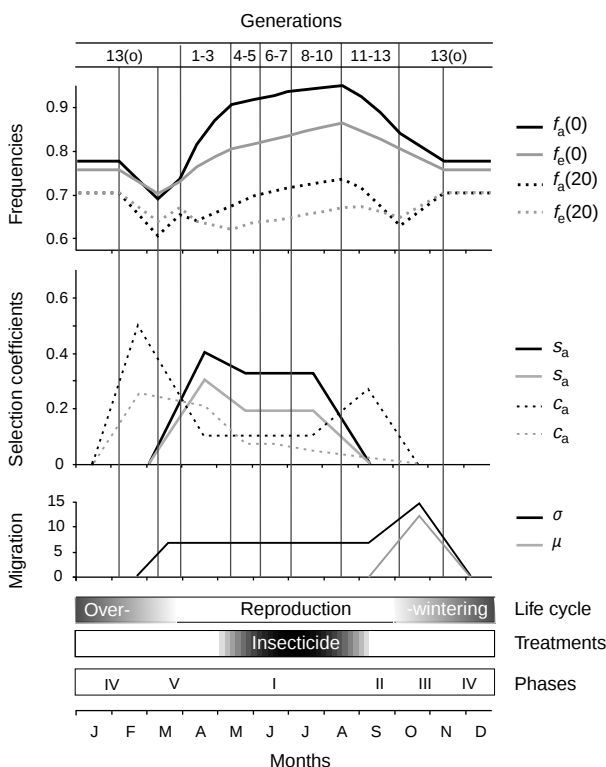


Figure 3 Annual variation of frequency clines at *Ace.1* (subscript a) and *Ester* (subscript e) fitted by the migration–selection model. These clines are represented by their frequency on the coast, $f(0)$, and at 20 km from the coast, $f(20)$. Corresponding seasonal variation of selection coefficients per generation due to insecticide treatments (s) and cost of resistance (c) as well as migration distribution mean (μ) and standard deviation (σ) in km are indicated. Vertical lines indicate dates of sampling. The number of generations considered between each of them is indicated on the top. The overwintering generation is indicated by (o) after the generation number. These estimates correspond to the best migration–selection model (that is, after model simplification). Each value represented is significantly different from zero and from other adjacent values on the graph of the same parameter. The cycle can be interpreted in five phases: insecticide treatment (I), end of the breeding season in the absence of treatment (II), migration to overwintering sites (III), overwintering (IV) and first spring generation (V).

19. Kettlewell, H. B. D. & Berry, R. J. The study of a cline. *Heredity* **16**, 403–414 (1961).
20. Mani, G. S. A theoretical study of morph ratio clines with special reference to melanism in moths. *Proc. R. Soc. Lond. B* **B210**, 299–316 (1980).
21. Jain, S. K. & Bradshaw, A. D. Evolutionary divergence among adjacent plant populations. I. The evidence and its theoretical analysis. *Heredity* **21**, 407–441 (1966).
22. Chevillon, C., Bourguet, D., Rousset, F., Pasteur, N. & Raymond, M. Pleiotropy of adaptive changes in populations: comparisons among insecticide resistance genes in *Culex pipiens*. *Genet. Res. Camb.* **70**, 195–204 (1997).
23. Guillemaud, T. *et al.* Evolution of resistance in *Culex pipiens*: allele replacement and changing environment. *Evolution* **52**, 443–453 (1998).
24. Lenormand, T., Guillemaud, T., Bourguet, D. & Raymond, M. Evaluating gene flow using selected markers: a case study. *Genetics* **149**, 1383–1392 (1998).
25. Lenormand, T., Guillemaud, T., Bourguet, D. & Raymond, M. Appearance and sweep of a gene duplication: adaptive response and potential for a new function in the mosquito *Culex pipiens*. *Evolution* **52**, 1705–1712 (1998).
26. Slatkin, M. Gene flow and selection in a two locus system. *Genetics* **81**, 787–802 (1975).
27. Lenormand, T. & Raymond, M. Analysis of clines with variable selection and variable migration. *Am. Nat.* (in the press).
28. Lenormand, T. & Raymond, M. Resistance management: the stable zone strategy. *Proc. R. Soc. Lond. B* **265**, 1985–1990 (1998).
29. Anderson, D. R., Burnham, K. P. & White, G. C. AIC model selection in overdispersed capture-recapture data. *Ecology* **75**, 1780–1793 (1994).

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Illusory shifts in visual direction accompany adaptation of saccadic eye movements

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A central problem in human vision is to explain how the visual world remains stable despite the continual displacements of the retinal image produced by rapid saccadic movements of the eyes. Perceived stability has been attributed to 'efferent-copy' signals, representing the saccadic motor commands, that cancel the effects of saccade-related retinal displacements^{1–6}. Here we show, by means of a perceptual illusion, that traditional cancellation theories cannot explain stability. The perceptual illusion was produced by first inducing adaptive changes in saccadic gain (ratio of saccade size to target eccentricity). Following adaptation, subjects experienced an illusory mislocalization in which widely separated targets flashed before and after saccades appeared to be in the same place. The illusion shows that the perceptual system did not take the adaptive changes into account. Perceptual localization is based on signals representing the size of the initially-intended saccade, not the size of the saccade that is ultimately executed. Signals representing intended saccades initiate a visual comparison process used to maintain perceptual stability across saccades and to generate the oculomotor error signals that ensure saccadic accuracy.

Saccadic adaptation was produced conventionally^{7–11}. Subjects made a single saccade to look at a target point that stepped abruptly to an eccentric horizontal location, 228, 240 or 252 arcmin away. The background was dark; only the target was visible. Saccades were accurate, as expected^{11,12} (Fig. 1a). In the adaptation trials, which began after 20 baseline step-tracking trials, the target hopped either forwards or backwards by 48 arcmin (about 20% of the size of the original step) during the saccade. (Forward and backward hops, and rightward and leftward saccades, were tested in separate experimental sessions.) Saccades landed near the original

(pre-hop) target location during the first few adaption trials (Fig. 1b). Adaptive changes occurred over the ensuing trials, when saccades began to land closer to the target's final, post-hop position (Fig. 1c).

After adaptation reached nearly asymptotic levels, 'probe' trials were introduced to assess the perceived relative location of targets flashed before and after the saccade^{13–17} (Fig. 1d). A probe trial was run after every three consecutive adaptation trials, thus allowing saccades to remain in the adapted state. The saccadic target in probe trials was flashed for 100 ms. The display remained dark during the saccade to the remembered location of the flash. Two-hundred-and-fifty milliseconds after saccade detection, the post-saccadic probe target was flashed for 100 ms. The subject reported whether the post-saccadic probe was located to the left or to the right of the pre-saccadic target. We used a double-random staircase procedure (see Methods) to select the post-saccadic probe location according to the response on the immediately preceding probe trial, with the goal of 'zooming in' on the location that perceptually matched the remembered location of the pre-saccadic target.

Figure 2 (open symbols) shows the mean sizes of saccades to the right and left over successive blocks of 20 trials for the three subjects (two naive; one author) during forward, backward and no-hop (control) sessions. Saccadic gain (saccade size/target step size) was similar across the three target step sizes (228, 240 and 252 arcmin) so data from the three step sizes was combined (see Methods). Saccadic adaptation reached maximum levels after about 20–60 adaptation trials. The maximum levels of adaptation were less than hop size, and dependent on subject, saccadic direction and hop

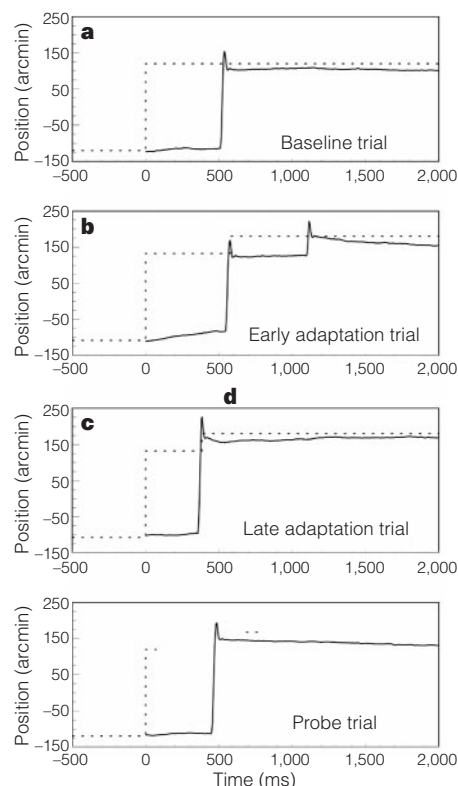


Figure 1 Representative traces of horizontal eye (solid line) and stimulus position (dotted line). Negative values are left of centre. **a**, Saccade in response to a 240-arcmin target step. Saccade size is the difference between pre- and post-saccadic eye position, bypassing the overshoot at the end of the saccade (see Methods). **b**, An early adaptation trial, 48-arcmin forward hop. **c**, A later adaptation trial. **d**, Probe trial, with pre- and post-saccadic flashes to be compared. Because of the intervening saccade, the pre-saccadic target and post-saccadic probe occupied different retinal loci.

direction. Overall, adaptive performance is typical of previous reports⁷⁻¹¹.

Perceptual localization during the same experimental sessions is shown by the average locations of the final 20 post-saccadic probes (filled symbols on right-hand ordinates). An accurate perceptual match of pre- and post-saccadic targets would be shown at 240 arcmin. Matches in the control (no-hop) sessions (circles) were typically accurate¹⁴⁻¹⁶. The illusory mislocalizations during the adaptation sessions are shown by the differences between post-saccadic probe locations in the adaptation (filled triangles) and no-hop sessions. The mislocalizations were in the same direction as the hops and in the same direction as the adaptive changes in saccades. That is, during forward-hop sessions, where saccadic amplitude adaptively increased, post-saccadic probes located further from the initial fixation position than the pre-saccadic target were seen to be in the same location as the pre-saccadic target. During backward-hop sessions, where saccadic amplitude adaptively decreased, post-saccadic probes located closer to the initial fixation position than the pre-saccadic target were seen to be in the same location as the pre-saccadic target. The illusion was found for all subjects and both saccadic directions. For subject E.K. the illusion was superimposed on a steady-state perceptual misalignment observed during no-hop sessions. Re-testing of the subjects under the same experimental conditions produced the same pattern of results.

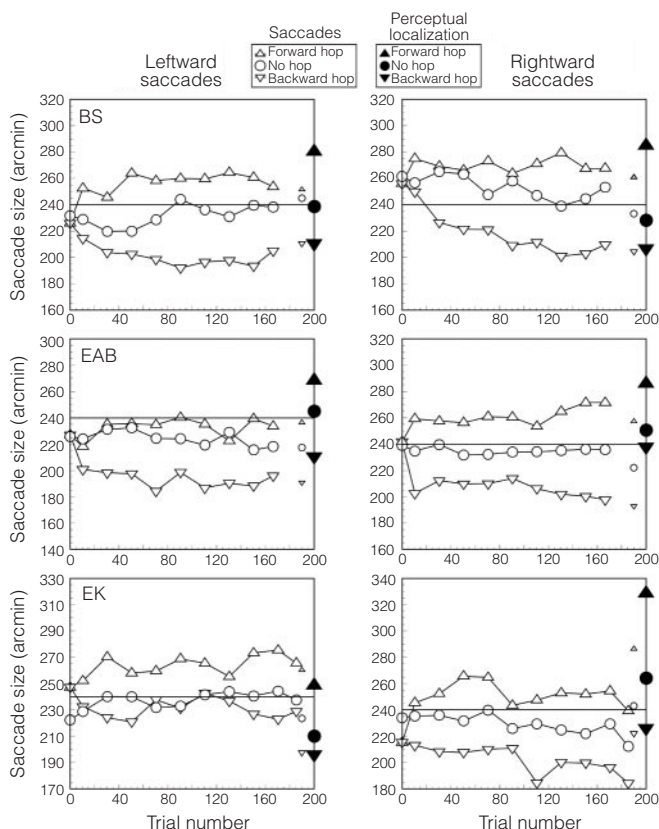


Figure 2 Saccadic adaptation and perceptual localization for each subject. Large open symbols: mean saccade size in blocks of 20 trials as a function of the middle trial in each block. Small open symbols: mean saccade size in probe trials. Data from the three target step sizes (228, 240 and 252 arcmin) were pooled (see Methods). Performance at trial zero is the average of 20 no-hop baseline trials. Standard errors are approximately the size of the large plotting symbols. Filled symbols on right-hand ordinate: post-saccadic probe location (average of final 10 probe trials per staircase; s.e. < 2.5 arcmin). An accurate match of the post-saccadic probe to the pre-saccadic target would be shown at 240 arcmin (horizontal line).

Figure 3 shows a consistent relationship between the magnitude and direction (forward versus backward) of saccadic adaptation and the magnitude and direction of the illusion. Discrepancies between adaptation and the illusion might be expected in light of the different sources of variability that affect motor performance and perceptual judgments¹⁷. Despite these differences, the correlation between saccadic adaptation and the illusion was 0.9, with most of the discrepancies being due to scatter in the magnitude of the illusion in sessions producing the largest amounts of saccadic adaptation.

In probe trials the display was blank for a brief period of time between the pre-saccadic target and the probe (Fig. 1d) to avoid presenting visual targets near the time of the saccade, when large errors in perceptual localization are expected^{13,15,16}. The blank interval was not essential. The illusion was also found when the pre-saccadic target remained on, hopping from its original location to the probe location during the saccade. The illusion was also present when subjects were instructed to saccade 75% of the way to the target in adaptation and probe trials. Saccadic adaptation and the illusion were abolished by briefly (400 ms) removing the target from view during adaptation trials as soon as the saccade was detected. The blank interval during adaptation trials made the hop more apparent¹⁸. When re-tested in the basic experiment after running adaptation trials containing the blank interval, subject B.S. no longer either adapted or experienced the illusion (the other two subjects continued to adapt and show the illusion). The cessation of adaptation for subject B.S. shows that the presence of the target hops in adaptation trials was not responsible for the illusion. The illusion was tied to the adaptive changes in the saccades.

The illusory misalignment of pre- and post-saccadic targets accompanying saccadic adaptation indicates that the perceptual system is not using a signal representing the actual size of the saccade. Had such a signal been available, pre- and post-saccadic targets would have been perceived as accurately aligned, independent of the adaptive state of the saccadic system. Instead, the illusory misalignment indicates that the perceptual system was unaware of the adaptive changes in saccadic magnitude. The pre- and post-saccadic targets were aligned according to a signal that corresponded to the magnitude of the original displacement of the target, and the originally intended saccade, rather than to the

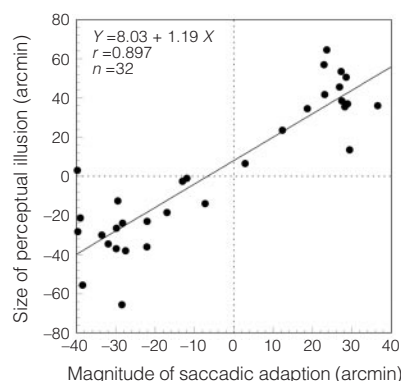


Figure 3 Magnitude of saccadic adaptation plotted against the size of the perceptual illusion. The magnitude of adaptation was estimated by the difference between average saccade size in the last 100 trials of the hop and no-hop sessions. The magnitude of the illusion was estimated by the difference between average probe locations (last 20 probe trials) in hop and no-hop sessions. Positive values indicate adaptation or localization forwards of the pre-saccadic target. The 32 points show data for two hop directions (forward, backward), two saccadic directions (left, right) and three replications for subjects E.A.B. and E.K. and two for subject B.S.

saccade that was actually made (see refs 14, 19 and 20 for related suggestions). Such a signal could be created by re-calibrating a representation of the final (post-adaptation) saccadic command. A simpler solution would be for the perceptual system to tap into the generation of the saccadic command at a high level, before the site of the adaptive changes. This high-level signal may be related to the efferent-copy signals that produce changes in neural activity²¹ and shifts in the receptive field locations²² of single neurons in parietal cortex.

What is the advantage of relying on a high-level efferent copy signal that is likely to be an imperfect representation of the actual saccade? The advantage may lie in the process of saccadic adaptation itself. Evidence indicates that saccadic adaptation is localized fairly late in the motor-processing stream, probably involving the cerebellum^{23–26}. A useful error signal for adaptation can be generated as a result of a dissociation between a high-level efferent copy signal, which predicts the content of the future foveal image, from the metrics of the executed saccade. With such a dissociation, any deviation of the actual from the predicted foveal image can be attributed to events at lower levels of the oculomotor system, downstream from the initial coding of the intended saccade. The failure to attain the predicted foveal image produces perceptual mislocalizations, such as those we observed, but, more importantly, it can provide an unambiguous error signal to trigger subsequent adaptive modifications of saccades. Adaptation would then continue until saccade size changed sufficiently so that the post-saccadic image matched, within some criterion, the pre-saccadic prediction.

The illusory mislocalizations accompanying saccadic adaptation show that the perceptual system is not making use of signals representing actual, executed saccades, as is often assumed^{1–6}. Signals representing initial saccadic intentions, generated before the site of adaptation, can initiate a visual comparison of pre- and post-saccadic images that would both contribute to stable percepts and ensure accurate saccades. □

Methods

Subjects. The subjects were E.A.B. and B.S., naive as to the purpose of the experiment, and E.K., an author. All had previous experience as eye-movement subjects.

Eye moment recording. Movements of the right eye, with the head stabilized, were recorded by a Generation IV SRI Double Purkinje Image Tracker (sensitivity <1 arcmin)²⁷. Tracker output was filtered (50 Hz) and sampled every 5 ms^{12,28}.

Stimulus. The stimulus was a single point, 2 log units above the absolute light-adapted foveal threshold, refreshed every 20 ms, shown on a Tektronix 608 display monitor (fast-decay P4 phosphor) located directly in front of the right eye. Except for the target point the room was totally dark. The stimulus was viewed through a collimating lens that placed it at optical infinity.

Adaptation trials. Subjects fixated a single point located either 108, 120 or 132 arcmin (selected randomly) to the left (for testing rightward saccades) or right (for leftward saccades) of straight ahead and started trials by pressing a button when ready. Two-hundred milliseconds later the target point stepped 228, 240 or 252 arcmin (selected randomly). Subjects aimed a single saccade at the pre-saccadic target, keeping the latency long enough to avoid compromising accuracy, and not trying to reach the target with a sequence of two or more saccades. These instructions produce accurate saccades and reduce extraneous behavioural variability^{12,28}. When the saccade was detected on-line (position change of 12–15 arcmin in 5 ms) the target hopped 48 arcmin forwards or backwards or not at all, and remained at its new location for the remainder of the 2-s trial. Each session began with 20 no-hop baseline trials followed by 172 adaptation trials for E.A.B. and B.S., and 190 for E.K. Forward, backward and no-hop trials, and rightward and leftward saccades were tested in separate sessions. The order of testing hop conditions within a replication (3 hop conditions × 2 saccadic directions) was random; saccadic direction (left or right) was alternated from session to session.

Perceptual probe trials. Probe trials (34 per session for E.A.B. and B.S. and 40

trials per session for E.K.) began after the first 70 adaptation trials and were tested after every third adaptation trial thereafter. The target for the saccade was flashed for 100 ms. The display was dark until 250 ms after saccade detection when a single point (the probe) was flashed for 100 ms. The subject pressed a button to indicate whether the probe was to the right or to the left of the initial target flash. A double random staircase procedure determined probe location²⁹. Specifically, if the subject indicated that the probe was to the left of the saccadic target, the next probe in the staircase would be shifted to the right; responses of 'right' shifted the next probe to the left. Probes were shifted by 15 arcmin for the first 5 probe trials, 10 arcmin for the next 7 trials and 5 arcmin thereafter. Two independent randomly interleaved staircases were run, one starting to the right of the saccadic target and the other to the left. Data were pooled over the three target step sizes by calculating localization error (probe location minus presaccadic target location) on each trial.

Analysis. Saccades were detected off-line by an acceleration criterion. Saccade size was the distance between the mean position of the eye at the start of the trial and the position at the end of the saccade, bypassing the overshoots^{12,28}. Overshoots are in part genuine characteristics of saccades and in part due to the Dual Purkinje Eyetracker's sensitivity to movements of the lens during saccades³⁰. Data from the three target step sizes (228, 240 and 252 arcmin) were pooled after multiplying saccadic gain (saccadic size/target step size) by the middle step (240 arcmin).

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1. von Helmholtz, H. *Treatise on Physiological Optics* vol. 3 (1910) (trans. Southall, J. P. C.) (Dover, New York, 1963).
2. von Holst, E. & Mittelstaedt, H. Das Reafferenzprinzip. *Naturwissenschaften* **37**, 464–476 (1950).
3. MacKay, D. M. in *Handbook of Sensory Physiology* vol. VII/3: Central Processing of Visual Information (ed. Jung, R.) 307–331 (Springer, Berlin, 1973).
4. Martin, L. in *Handbook of Sensory Physiology* vol. VII/4: Visual Psychophysics (eds Jameson, D. & Hurvich, L. M.) 331–380 (Springer, Berlin, 1972).
5. Skavenski, A. A., Haddad, G. M. & Steinman, R. M. The extraretinal signal for the visual perception of direction. *Percept. Psychophys.* **11**, 287–290 (1972).
6. Skavenski, A. A. in *Eye Movements and Their Role in Visual and Cognitive Processes* (ed. Kowler, E.) 263–287 (Elsevier, Amsterdam, 1990).
7. McLaughlin, S. C. Parametric adjustment in saccadic eye movement. *Percept. Psychophys.* **2**, 359–362 (1967).
8. Deubel, H. Separate adaptive mechanisms for the control of reactive and volitional saccadic eye movements. *Vision Res.* **35**, 3529–3540 (1995).
9. Miller, J. M., Anstis, T. & Templeton, W. B. Saccadic plasticity: parametric adaptive control by retinal feedback. *J. Exp. Psychol. Hum. Percept. Perform.* **7**, 356–366 (1981).
10. Erkelens, C. J. & Hulleman, J. Selective adaptation of internally triggered saccades made to visual targets. *Exp. Brain Res.* **93**, 157–164 (1993).
11. Carpenter, R. H. S. *Movements of the Eyes* 2nd edn (Pion Press, 1992).
12. Kowler, E. & Blaser, E. The accuracy and precision of saccades to small and large targets. *Vision Res.* **35**, 1741–1754 (1995).
13. Martin, L. & Pearce, D. G. Visual perception of direction for stimuli flashed during voluntary saccadic eye movement. *Science* **165**, 1485–1488 (1965).
14. Lennie, P. & Siddle, A. Saccadic eye movements and visual stability. *Nature* **275**, 766–768 (1978).
15. Dassonville, P., Schlag, J. & Schlag-Rey, M. Oculomotor localization relies of a damped representation of saccadic eye displacement in human and nonhuman primates. *Vis. Neurosci.* **9**, 261–269 (1992).
16. Sperling, G. in *Eye Movements and Their Role in Visual and Cognitive Processes* (ed. Kowler, E.) 307–351 (Elsevier, Amsterdam, 1990).
17. Goodale, M. A., Pelisson, D. & Prablanc, C. Large adjustments in visually guided reaching do not depend on vision of the hand or perception of target displacement. *Nature* **320**, 748–750 (1986).
18. Deubel, H., Schneider, W. X. & Bridgeman, B. Postsaccadic target blanking prevents saccadic suppression of image displacement. *Vision Res.* **36**, 985–996 (1996).
19. Ludvig, E. Control of ocular movements and visual interpretation of environment. *AMA Arch. Ophthalmol.* **48**, 442–448 (1952).
20. Pola, J. in *Eye Movements and Psychological Processes* (eds Monty, R. A. & Senders, J. W.) 245–254 (Erlbaum, Hillsdale, New Jersey, 1976).
21. Andersen, R. A., Essick, G. K. & Siegel, R. M. Encoding spatial location by posterior parietal neurons. *Science* **230**, 456–458 (1985).
22. Duhamel, J.-R., Colby, C. L. & Goldberg, M. E. The updating of the representation of visual space in parietal cortex by intended eye movements. *Science* **255**, 90–92 (1992).
23. Optican, L. M. in *Adaptive Mechanisms in Gaze Control* (eds Berthoz, A. & Melvill-Jones, G.) 71–79 (Elsevier, Amsterdam, 1985).
24. Goldberg, M. E., Musil, S. Y., Fitzgibbon, E. J., Smith, M. & Olson, C. R. in *Role of the Cerebellum and Basal Ganglia in Voluntary Movements* (eds Mano, M., Hamada, I. & DeLong, M. R.) 203–211 (Elsevier, Amsterdam, 1993).
25. Frens, M. A. & Van Opstal, A. J. Monkey superior colliculus activity during short-term saccadic adaptation. *Brain Res. Bull.* **43**, 473–483 (1997).
26. Desmurget, M. et al. Functional anatomy of saccadic adaptation in humans. *Nature Neurosci.* **1**, 524–528 (1998).
27. Crane, H. D. & Steele, C. S. Accurate three-dimensional eyetracker. *Appl. Opt.* **17**, 691–705 (1978).
28. McGowan, J., Kowler, E., Sharma, A. & Chubb, C. Saccadic localization of random dot patterns. *Vision Res.* **38**, 895–909 (1998).
29. Cornsweet, T. The staircase method in psychophysics. *Am. J. Psychol.* **75**, 485–491 (1962).
30. Leigh, R. J. & Zee, D. S. *The Neurobiology of Eye Movements* 82 (F. A. Davis, Philadelphia, 1991).

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Electrophysiological measurement of rapid shifts of attention during visual search

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The perception of natural visual scenes that contain many objects poses computational problems that are absent when objects are perceived in isolation¹. Vision researchers have captured this attribute of real-world perception in the laboratory by using visual search tasks, in which subjects search for a target object in arrays containing varying numbers of non-target distractor objects. Under many conditions, the amount of time required to detect a visual search target increases as the number of objects in the stimulus array increases, and some investigators have proposed that this reflects the serial application of attention to the individual objects in the array^{2,3}. However, other investigators have argued that this pattern of results may instead be due to limitations in the processing capacity of a parallel processing system that identifies multiple objects concurrently^{4,5}. Here we attempt to address this longstanding controversy by using an electrophysiological marker of the moment-by-moment direction of attention—the N2pc component of the event-related potential waveform—to show that attention shifts rapidly among objects during visual search.

Behavioural studies of visual search have not conclusively resolved this dispute because they do not provide a moment-by-moment measure of the spatial distribution of attention, making it impossible to determine whether attention shifts rapidly from item to item. In contrast, the N2pc component of the event-related potential (ERP) waveform provides a continuous measure of the distribution of attention by virtue of its lateralized distribution. Specifically, N2pc is a negative-going voltage deflection that is typically observed 200–300 ms after the onset of a visual search array, and it is largest over areas of visual cortex in the hemisphere contralateral to the location of an attended object within the search array. Several experiments have shown that the N2pc component is related to the covert orienting of visual attention before the completion of object recognition^{6,7}, and further experiments have shown that it reflects a spatial filtering process that closely resembles attention-related modulations of activity measured from cortical neurons in monkeys⁸. Thus, if visual search involves rapid, serial shifts of attention, the N2pc component should shift rapidly between the left and right hemispheres as attention shifts rapidly between the right and left hemifields.

In standard visual search protocols, it is impossible to determine the order in which objects are searched, and this in turn makes it impossible to distinguish between attention-related shifts in the N2pc component and random voltage fluctuations. To solve this problem, we used a modified visual search paradigm in which the subjects were biased to search the objects in a known order. Each search array contained four coloured squares, one in each quadrant, along with 21 black distractor squares (Fig. 1a). The target, a square with a gap on the left side, was present on 50% of trials. To bias the order in which the squares might be searched, we presented the target in one prespecified colour on 75% of the target-present trials and in another prespecified colour on the remaining 25%. We refer to these colours as C_{75} and C_{25} , respectively. For example, to bias a subject to search the red item first and the green item second, the target for that subject was red on 75% of target-present trials and green on 25% of target-present trials. The subjects were informed of the probabilities at the beginning of the session so that they would

be familiar with the probability structure. However, they were given no instructions about how to use the probability information (they were not told to search C_{75} first).

Subjects detected the target about 80 ms faster when it was the C_{75} item ($M = 645$ ms) than when it was the C_{25} item ($M = 723$ ms), a statistically significant difference ($P < 0.001$). This result is consistent with both serial and parallel models of attention, but these models make differing predictions concerning the N2pc component. When C_{75} and C_{25} were in opposite hemifields, serial models would predict that the N2pc component would appear first over the hemisphere contralateral to C_{75} and then shift to the hemisphere contralateral to C_{25} (except when the C_{75} item was the target, in which case attention would remain focused on this item). In contrast, typical parallel models would predict that the N2pc component would not switch rapidly between the hemispheres, but would instead be consistently larger over the hemisphere contralateral to C_{75} , owing to a greater allocation of processing resources to this item. Thus, serial models predict rapid interhemispheric switching of the N2pc component whereas typical parallel models predict no change over time.

Figure 1b shows the N2pc component on target-absent trials when C_{75} and C_{25} were in opposite hemifields. As predicted by serial search models, the N2pc was more negative over the hemisphere contralateral to C_{75} from 200–300 ms post-stimulus and then became more negative over the hemisphere contralateral to C_{25} from 300–450 ms post-stimulus. When C_{25} and C_{75} were both in the

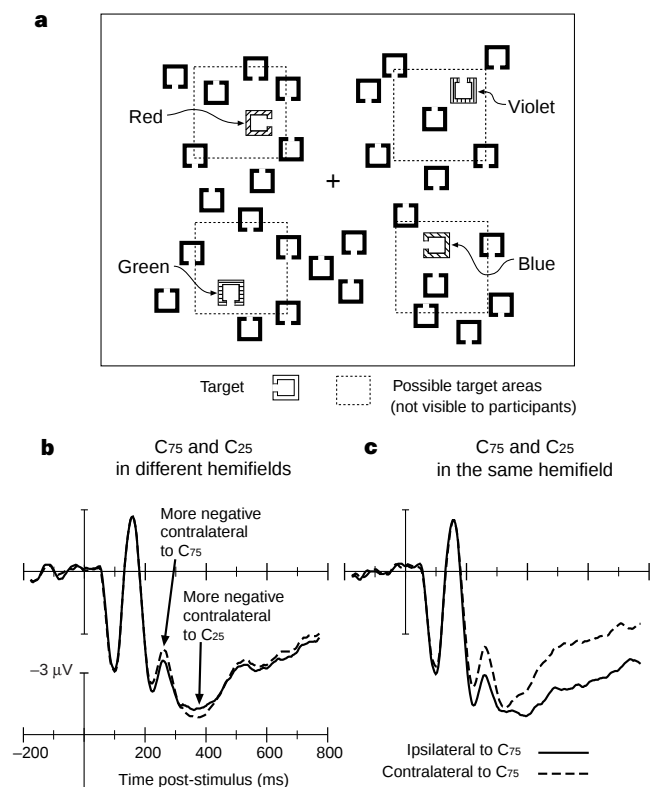


Figure 1 Stimuli and results from the first experiment. **a**, Example visual search array. **b**, ERP waveforms for non-target stimuli at lateral occipital electrode sites when C_{75} and C_{25} were in opposite hemifields, averaged across subjects. The contralateral waveform is an average of the left hemisphere waveform when C_{75} was in the right visual field and the right hemisphere waveform when C_{75} was in the left visual field, and the ipsilateral waveform is an average of the left hemisphere waveform when C_{75} was in the left visual field and the right hemisphere waveform when C_{75} was in the right visual field. **c**, Analogous waveforms when C_{75} and C_{25} were in the same hemifield.

same hemifield (Fig. 1c), the N2pc remained contralateral to that hemifield until the end of the recording epoch (consistent with both serial and parallel models). Thus, the 80-ms difference in response time between the C_{75} and C_{25} targets was paralleled by a similarly timed shift in N2pc lateralization when C_{75} and C_{25} were in opposite hemifields. These results show that, at least under some conditions, attention shifts rapidly among the non-target items in a visual search array. Moreover, the timing of the shift is comparable to the search rates obtained in behavioural experiments with similarly difficult-to-discriminate targets⁹.

To assess these results statistically, N2pc measurements for early (200–275 ms) and late (350–425 ms) time intervals were entered into an analysis of variance (ANOVA) with factors of stimulus configuration (that is, C_{75} and C_{25} in the same versus opposite hemifields), N2pc measurement interval and electrode site. The presence of a polarity reversal between the early and late intervals when C_{75} and C_{25} were in opposite hemifields, but not when they were in the same hemifield, led to a statistically significant interaction between stimulus configuration and N2pc measurement interval ($F[1, 9] = 5.68$, $P < 0.05$). Additional analyses confirmed that, when C_{75} and C_{25} were in opposite hemifields, the N2pc effects were significant in both the early and late intervals ($F[1, 9] = 8.40$, $P < 0.02$ and $F[1, 9] = 5.88$, $P < 0.05$, respectively). In addition, to test whether the early and late phases of the N2pc component in Fig. 1b arose from the same neural generator source, normalized scalp distributions¹⁰ were compared in an additional ANOVA (including only the data from trials in which C_{75} and C_{25} were in opposite hemifields). The interaction between electrode site and N2pc measurement interval in this ANOVA was very far from statistical significance ($F[11, 99] = 0.50$, $P = 0.90$), consistent with a common neural generator source.

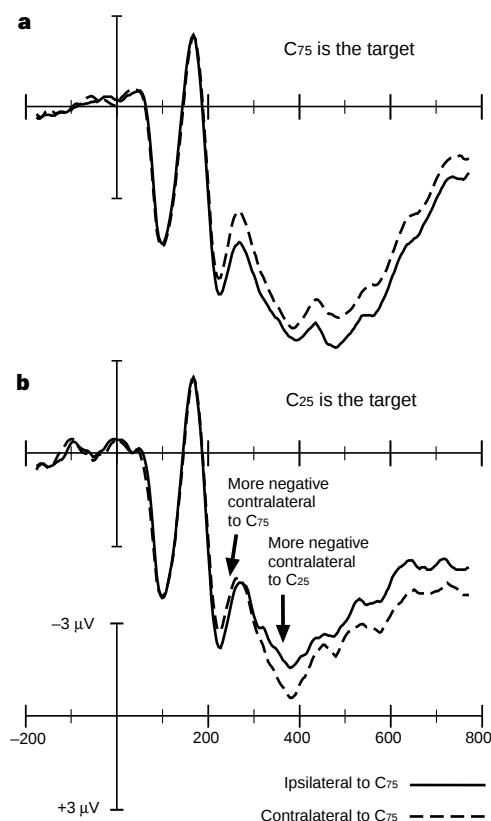


Figure 2 ERP waveforms at lateral occipital electrode sites when C_{75} and C_{25} were in opposite hemifields, averaged across subjects for C_{75} targets (a) and C_{25} targets (b) in the second experiment.

There were too few C_{25} targets in this experiment to provide an adequate analysis of the target-present trials, and a second experiment was therefore conducted in which a target was present on every trial. Subjects reported whether the target's gap was on the top or bottom of the square (non-targets had a left gap or a right gap). When C_{75} was the target, the N2pc component was more negative contralateral to this item from 200 ms until the end of the recording epoch (Fig. 2a). In contrast, when C_{25} was the target, the N2pc was initially more negative contralateral to C_{75} and then became more negative contralateral to C_{25} (Fig. 2b). This pattern of effects led to a statistically significant interaction between target item (C_{75} versus C_{25}) and N2pc measurement interval ($F[1, 9] = 7.77$, $P < 0.02$). Additional analyses confirmed that the N2pc effects for the C_{75} targets were significant in both the early and late intervals ($F[1, 9] = 6.49$, $P < 0.05$ and $F[1, 9] = 5.33$, $P < 0.05$, respectively). These results indicate that attention was first directed toward C_{75} and, if the visual system determined that this item was not the target, attention was then rapidly redirected toward C_{25} .

It is possible that the probability manipulation that we used to bias the search order in these experiments led subjects to adopt a serial search strategy even though they would ordinarily use a parallel strategy. A third experiment was therefore conducted that did not use an artificial means of biasing the search order but instead took advantage of subjects' pre-existing tendencies to search items near fixation before searching items far from fixation^{11,12}. The search arrays in this experiment consisted of 40 black items and 1 or 2 red items, with the red items occurring either near or far from fixation and in the same or opposite hemifields. The target, if present, was always a red item and was equally likely to appear at either eccentricity.

As shown in Fig. 3, when one red item was near fixation and the other was far from fixation and in the opposite hemifield, the N2pc was more negative contralateral to the near item from about 200–300 ms and then become more negative contralateral to the far item from about 300–450 ms. This reversal was not present when the near and far items were in the same hemifield, and these different patterns of lateralization led to a statistically significant interaction between stimulus configuration (near and far items in the same versus opposite hemifields) and N2pc measurement interval ($F[1, 9] = 7.72$, $P = 0.02$). Additional analyses confirmed that the N2pc effects shown in Fig. 3 were significant in both the early and late intervals ($F[1, 9] = 7.03$, $P < 0.05$ and $F[1, 9] = 6.80$, $P < 0.05$, respectively).

This experiment indicates that attention switches rapidly among the non-target items even when no artificial methods are used to

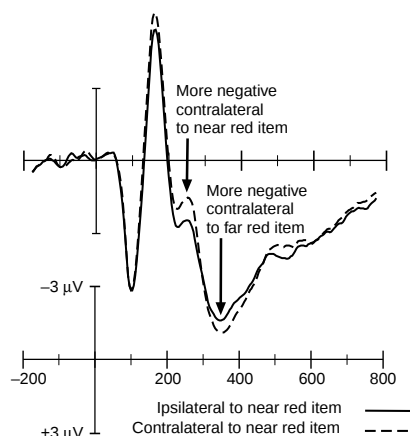


Figure 3 ERP waveforms for non-target stimuli at lateral occipital electrode sites when the near and far red items were in opposite hemifields, averaged across subjects in the third experiment.

bias the search order, consistent with serial models of visual search. However, this pattern of results could potentially be explained by an intrinsically slower N2pc time course for the far item than for the near item. To rule out this possibility, we included trials with a single red item, and the N2pc on these trials had almost exactly the same time course whether this item was near or far. Thus, the results shown in Fig. 3 cannot be explained by intrinsic differences in the time course of processing for near and far items.

A completely flexible parallel model can emulate any serial model¹³, and it is therefore impossible to rule out all parallel search models without also ruling out all serial search models. For example, 75% of processing resources might initially be allocated to the C₇₅ item, and when the visual system has determined that this item is not the target, these resources might be rapidly released to the C₂₅ item, producing results such as those obtained here. However, the present results do rule out the vast majority of parallel models in which the overall distribution of attention does not shift rapidly among the items in the search array; that is, those that do not approximate a serial search model. Thus, by electrophysiologically tracking the moment-by-moment distribution of attention, we have provided direct evidence that visual search involves rapid shifts in the distribution of attention among objects. □

Methods

Stimulus arrays in the first experiment were presented within a 9.8° × 9.8° region on a light grey background (9.9 cd m⁻²). Each item within an array subtended 0.72° × 0.72°, with a 0.2° gap on one side, and the items were presented in black, green, red, blue or violet. Arrays were presented for 2,000 ms, followed by a blank interstimulus interval of 800–1200 ms. In the second and third experiments, the sizes of the arrays and the individual items were reduced by 50% to minimize eye movements. In the first two experiments, each quadrant contained 5 or 6 black items and one coloured item, and the assignment of colours to quadrants varied randomly across trials. The specific colours used for C₇₅ and C₂₅ were randomized across subjects. In the third experiment, 10–11 items appeared in each quadrant, with either one or two red items in the array. When two red items were present, they were in different quadrants, selected at random; one was near fixation (0.6–1.0°) and the other was far from fixation (1.7–2.4°). As in previous studies of eccentricity in visual search, the sizes of the objects in the third experiment were increased at greater eccentricities according to the cortical magnification factor^{11,12}. Each subject received one training block and either 10 (first and second experiments) or 12 (third experiment) experimental blocks. Each block contained 104 trials.

ERPs were recorded from 10 neurologically normal college students in each experiment using our standard recording and analysis procedures⁷, including rejection of trials contaminated by blinks or large (>1°) eye movements. In addition, signal-averaged electro-oculogram recordings were used to ensure that average eye position did not deviate more than 0.2° toward the target for any subject. The N2pc was measured at occipital, lateral occipital and posterior temporal electrode sites as the difference in mean amplitude between the contralateral and ipsilateral waveforms, with measurement windows of 200–275 and 350–425 ms.

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1. Mozer, M. C. *The Perception of Multiple Objects* (MIT Press, Cambridge, Massachusetts, 1991).
2. Treisman, A. Features and objects: The fourteenth Bartlett memorial lecture. *Q. J. Exp. Psychol.* **40**, 201–237 (1988).
3. Wolfe, J. M. Guided search 2.0: A revised model of visual search. *Psychonomic Bull. Rev.* **1**, 202–238 (1994).
4. Bundesen, C. A theory of visual attention. *Psychol. Rev.* **97**, 523–547 (1990).
5. Duncan, J., Ward, R. & Shapiro, K. Direct measurement of attentional dwell time in human vision. *Nature* **369**, 313–315 (1994).
6. Luck, S. J. & Hillyard, S. A. Electrophysiological correlates of feature analysis during visual search. *Psychophysiology* **31**, 291–308 (1994).
7. Luck, S. J. & Hillyard, S. A. Spatial filtering during visual search: Evidence from human electrophysiology. *J. Exp. Psychol. Hum. Percept. Perform.* **20**, 1000–1014 (1994).
8. Luck, S. J., Girelli, M., McDermott, M. T. & Ford, M. A. Bridging the gap between monkey neurophysiology and human perception: An ambiguity resolution theory of visual selective attention. *Cogn. Psychol.* **33**, 64–87 (1997).
9. Wolfe, J. M. What can 1 million trials tell us about visual search? *Psychol. Sci.* **9**, 33–39 (1998).
10. McCarthy, G. & Wood, C. C. Scalp distributions of event-related potentials: An ambiguity associated with analysis of variance models. *Electroencephalogr. Clin. Neurophysiol.* **62**, 203–208 (1985).
11. Carrasco, M., Evert, D. L., Chang, I. & Katz, S. M. The eccentricity effect: Target eccentricity affects performance on conjunction searches. *Percept. Psychophys.* **57**, 1241–1261 (1995).

12. Wolfe, J. M., O'Neill, P. & Bennett, S. C. Why are there eccentricity effects in visual search? Visual and attentional hypotheses. *Percept. Psychophys.* **60**, 140–156 (1998).
13. Townsend, J. T. Serial vs. parallel processing. Sometimes they look like Tweedledum and Tweedledee but they can (and should) be distinguished. *Psychol. Sci.* **1**, 46–54 (1990).

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Global and fine information coded by single neurons in the temporal visual cortex

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When we see a person's face, we can easily recognize their species, individual identity and emotional state. How does the brain represent such complex information? A substantial number of neurons in the macaque temporal cortex respond to faces^{1–12}. However, the neuronal mechanisms underlying the processing of complex information are not yet clear. Here we recorded the activity of single neurons in the temporal cortex of macaque monkeys while presenting visual stimuli consisting of geometric shapes, and monkey and human faces with various expressions. Information theory was used to investigate how well the neuronal responses could categorize the stimuli. We found that single neurons conveyed two different scales of facial information in their firing patterns, starting at different latencies. Global information, categorizing stimuli as monkey faces, human faces or shapes, was conveyed in the earliest part of the responses. Fine information about identity or expression was conveyed later, beginning on average 51 ms after global information. We speculate that global information could be used as a 'header' to prepare destination areas for receiving more detailed information.

We recorded the activity of 1,874 single neurons in the inferior temporal cortex, including both banks of the superior temporal sulcus (STS), from A14 to A24, of four hemispheres in two monkeys (*Macaca fuscata*) (Fig. 1a). The monkeys were trained to maintain their gazes while visual stimuli were presented. The test stimuli (Fig. 1b–d) were coloured pictures of 16 monkey faces (4 models with 4 expressions), 12 human faces (3 models with 4 expressions) and 10 geometric shapes (rectangles and circles, each in 1 of 5 colours; brown is not shown in the figure). Of the 1,874 cells, 158 (8%) were face-responsive neurons, responding to at least one of the facial stimuli, of which 86 cells were quantitatively analysed.

As shown in Fig. 1b–d, a single face-responsive neuron responded to all facial stimuli but not to the shapes. Its temporal discharge patterns differed among the facial stimuli. For example, the full open-mouthed face (C) of all monkey models elicited strong and sustained discharges lasting up to 500 ms, whereas the neutral (A) and pout-lips (B) face of all monkey models elicited only the initial transient discharge. To determine how facial information was coded by the neuronal responses, the information was classified into one global and four fine categories. A global category (G) represents

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the stimulus groups monkey, human and shape. The four fine categories were classifications within the facial stimuli: F1, monkey identity; F2, monkey expression; F3, human identity; and F4, human expression. For each category, we calculated the time course of the transmitted information (I_c) from the number of spike discharges using a moving time window of 50 ms, and evaluated significance of information with the χ^2 test¹³ (see Methods).

Figure 2a shows the results of the information analysis for the responses of the neuron in Fig. 1. The neuron coded 'significant' information about all five categories tested. The earliest information was global (G). Its transmission rate increased rapidly, corresponding to the initial part of the averaged response (beige histogram), and then decreased. Information regarding F2 (monkey expression)

and F3 (human identity) peaked after the global peak. Once F2 peaked, it declined slowly and lasted during the sustained discharges, whereas global and F3 information fell rapidly from their peaks before levelling off. F1 (monkey identity) and F4 (human expression) were encoded at a much lower level than the others. The response latency of this neuron was 53 ms, and the latencies for the information about each category were as follows: G, 45; F1, 157; F2, 93; F3, 125; and F4, 125 ms. Thus, the earliest transient discharge conveyed global information, and the later sustained discharge encoded one category of the fine information best¹⁴.

Of the 86 face-responsive neurons, 11 neurons (13%) did not encode significant information in any category, 43 (50%) coded either global or fine category information, and 32 (37%) coded both categories. To clarify how single neurons encoded both global and

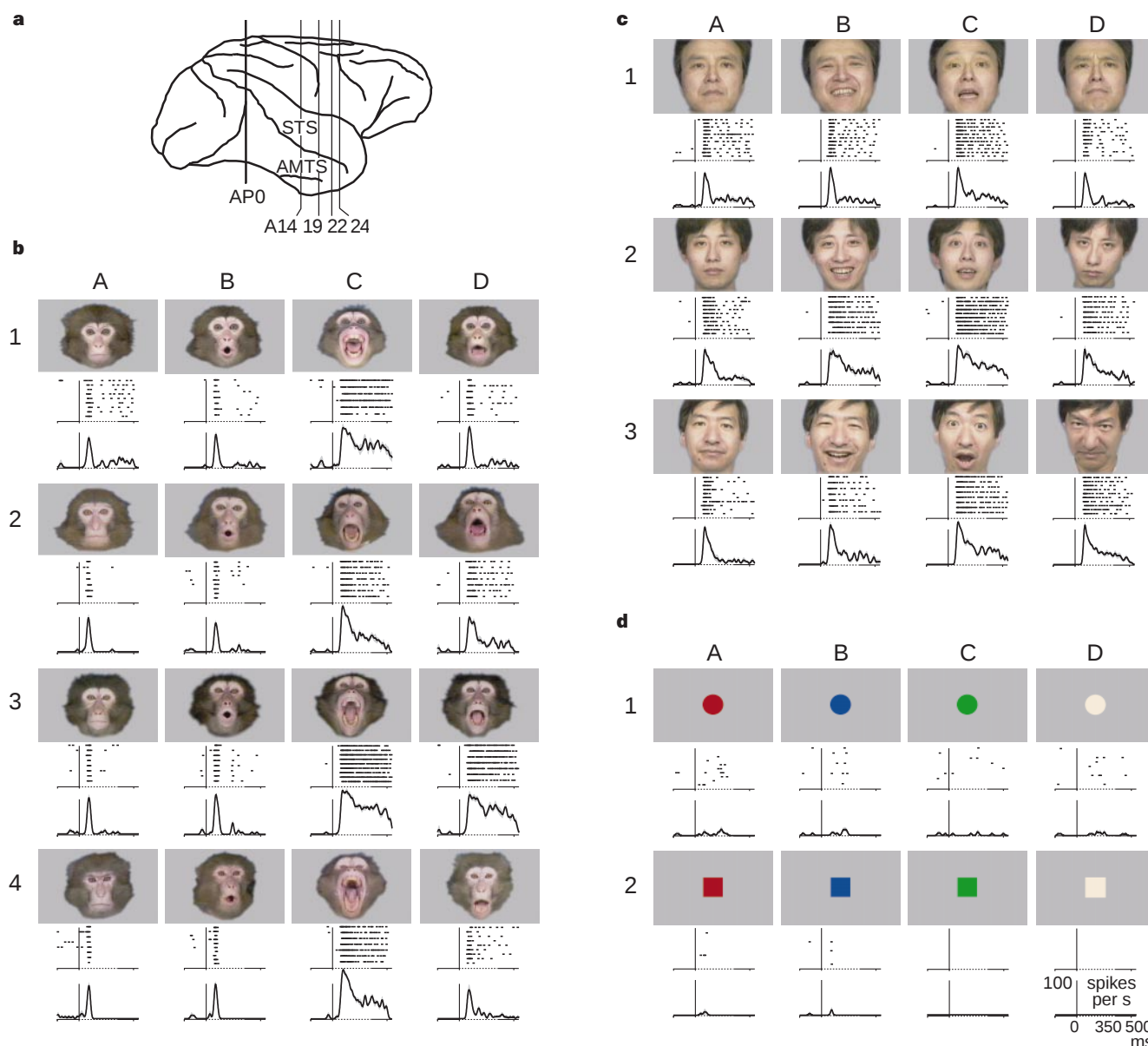


Figure 1 Responses of a face-responsive neuron. **a**, Areas of brain examined. AP0 represents the position of the external auditory meatus; A14, A19, A22 and A24 represent anterior 14, 19, 22 and 24 mm, respectively. AMTS, anterior middle temporal sulcus; STS, superior temporal sulcus. **b–d**, Response diagrams of a single neuron for monkey, human and shape stimuli, respectively. Each diagram consists of a stimulus image, a raster plot of the response and a spike-density plot, in the first, second and third rows, respectively. The expressions of 4 monkey

models were neutral (A), pout-lips (B), full open-mouthed (C) and mid open-mouthed (D). Those of 3 human models were neutral (A), happy (B), surprised (C) and angry (D). The colours of circles and rectangles were red (A), blue (B), green (C) and pink (D). For the spike-density plot, spikes per ms over all trials were summed and smoothed with a gaussian filter (s.d. = 10 ms). The vertical line in each plot indicates the time of stimulus onset, and the dashed part in the abscissa indicates the duration of stimulus presentation.

fine categories, we first compared the transmission rate between the two categories over the stimulus-presentation period. Global information was analysed for each of the 32 neurons. As some neurons coded more than one fine category, for the 32 neurons, a total of 56 cases of fine categories were analysed (F1, 18; F2, 22; F3, 11; and F4, 5). Figure 2b shows the summed I_c curves for the global (red line) and fine categories (black line), which aligned at the stimulus onset. Each thin line represents the successive sum of contributions from additional cases and each bold line shows the sum of all cases. The summed I_c curve for global information rose earlier than that of the fine categories. Among the neurons, the one in Fig. 2a exhibited the largest peak of information for both global (0.82 bits per 50 ms) and fine categories (F2, 0.81 bits per 50 ms). The averaged peak of the information for each category was as follows: G, 0.31 ± 0.20 ; F1, 0.14 ± 0.09 ; F2, 0.22 ± 0.16 ; F3, 0.25 ± 0.12 ; and F4, 0.16 ± 0.09 (mean $\pm$ s.d. in bits per 50 ms).

On the other hand, the average latency of the information for each category was as follows: G, 91 ± 33 ; F1, 154 ± 38 ; F2, 137 ± 52 ; F3, 150 ± 35 ; and F4, 181 ± 87 ms. The latency for global information differed significantly from those for F1 to F4 ($P < 0.0001$, Mann–Whitney test). We investigated whether this latency difference was due to differences in total uncertainty between global ($P = 0.33$) and fine discrimination ($P = 0.25$ for

F1, F2 and F4; $P = 0.33$ for F3) by measuring the information of F2 with two selected from the four monkey expressions ($P = 0.5$). Even with this simple condition, there was no significant information regarding the fine category in the early part of any response set. Subsequently, for each neuron, the information latency difference between categories was calculated by subtracting the latency of global information from those of the fine categories. When a neuron coded more than two fine categories the shortest latency was used as the latency of the fine category. The information latency for the fine category was an average of 51 ms longer than that of the global category for the 32 neurons (s.d., 39 ms; range, -8 to 128 ms; $P < 0.0001$, sign test). Thus, the global category preceded the fine category. Furthermore, global information was delayed by only 13 ms on average from the beginning of the neuronal response (s.d., 18 ms; range, -16 to 72 ms), indicating that the initial part of the neuronal response transmits global information.

The shorter latency of global information compared with the fine categories might be caused merely by the small response amplitude to the shape stimuli, as face-responsive neurons primarily responded to the faces. To examine this possibility, we measured the information related to another global category (G', monkey versus human) instead of G (monkey versus human versus shape). The information latency of G' was also shorter than that of the fine categories (F; 37 ± 35 , -16 to 112 ms; $P < 0.0001$, sign test). The latency difference between G' and F did not differ significantly from that between G and F for each neuron ($P = 0.21$, Mann–Whitney U test). Thus, whether the responses to the shapes were analysed does not affect the shorter information latencies of the global category. There is another possibility: that the peaks of both G and F occurred at approximately the same time, but the onset of global information might be measured as being earlier, as the peak of global information was greater than that of F. However, not only the latencies but also the peaks of global information were found to be earlier than those of F (G, 152 ± 57 ; F, 179 ± 49 ms; $P = 0.002$, Mann–Whitney U test). These analyses confirm that global information was conveyed in the neuronal response earlier than fine information.

As observed by others^{4–6,9,12}, the face-responsive neurons in the present study were located in both banks of the STS and in the inferior temporal gyrus from A14 to A24. Neurons conveying both global and fine information were recorded mainly in the more anterior part of the inferior temporal cortex (the anterior part of area TE), from A19 to A22 (Fig. 1a), and were distributed equally in the ventral surface of the inferior temporal gyrus and the lower bank of the STS. These are the regions where high stimulus-selectivity has been reported^{4–6,9}. The analyses in those studies, however, were mainly performed using averaged spike counts. Nonetheless, in the upper bank of the STS, it has been reported that different rotations of a face are discriminated instantaneously after response onset¹⁰, and movement direction of a head was processed before its rotation¹¹. Furthermore, in the inferior temporal cortex, a large amount of information about each facial stimulus exists within 100–200 ms after stimulus presentation¹². We found that the two different aspects of facial information, global and fine, reached their highest transmission rates at 117 and 165 ms, respectively (Fig. 2b). It has been shown that the time course of the information about two-dimensional monochrome patterns shows two peaks with a 94-ms interval between the information latency and the second peak¹⁵. Here the fine information reached a peak an average of 95 ms after the global information latency. So, in processing a variety of visual stimuli, the encoding of different information with a time delay may be common. The time delay might permit intra-areal contribution and feedback from other areas. Anatomically, the inferior temporal cortex connects reciprocally with more posterior parts of the inferior temporal cortex, the peri-rhinal and parahippocampal cortex^{16,17}, the parietal cortex^{18,19}, the orbitofrontal and prefrontal cortex¹⁹ and several subcortical areas, such as the amygdala²⁰. In the amygdala, a structure involved in social behaviour and emotion,

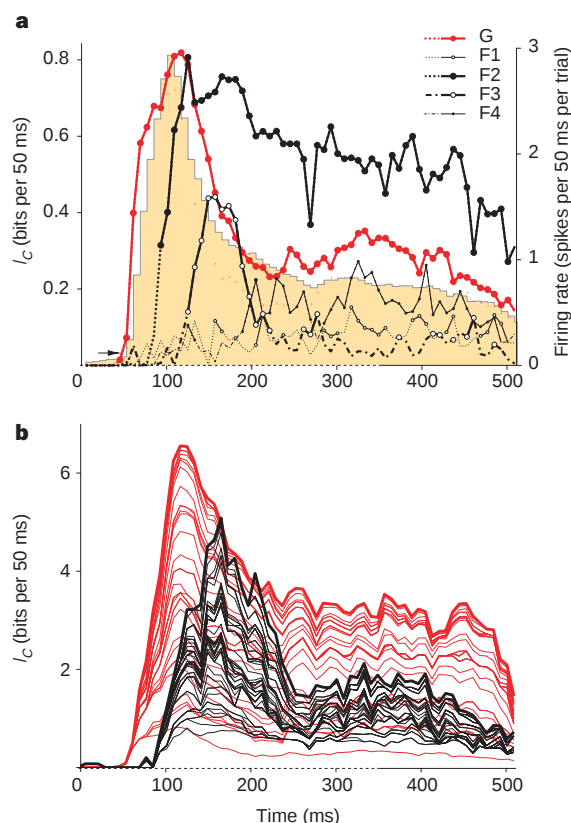


Figure 2 Information transmission rate (I_c) of face-responsive neurons. **a**, I_c curves and the averaged response (beige histogram) of a neuron in Fig. 1. I_c is plotted against the centre of the 50-ms sliding window. Solid and broken traces indicate significant and non-significant information, respectively. An arrow indicates the threshold criterion for the response latency. Each category contains the following number of trials: G (127, 115 and 90 trials for monkey, human and shape, respectively); F1 (30, 31, 32 and 34 for model 1, 2, 3 and 4); F2 (33, 35, 29 and 30 for A, B, C and D); F3 (43, 36 and 36 for model 1, 2 and 3); and F4 (31, 26, 30 and 28 for A, B, C and D). **b**, Summation of I_c curves for global (red) and fine categories (black) from 32 neurons aligned at the stimulus onset. Non-significant information was excluded from the analysis. In the time axis (abscissa), the time of stimulus onset is 0 ms and the duration of stimulus presentation is indicated as a dashed line.

some neurons process global information, and other neurons represent identity or expression of faces²¹. These amygdala neurons may interact with the face-responsive neurons in the inferior temporal cortex.

In various regions receiving axons from the inferior temporal cortex, early global information could serve as a 'header' to enhance subsequent processing by switching the processing mode. Multiplexing information in the codes from single neurons allows representation of more complex information than univariate codes. This type of sequential processing offers theoretical advantages for distinguishing among individual members of a single category²². Based on psychological results, a system of sequential processing of faces²³ has been proposed to explain the deficits seen in patients impaired only for differentiating faces (prosopagnosia²⁴), and the system has been expected to help in distinguishing among a large number of familiar people. Our result demonstrates a physiological precursor for a neural system that could carry out categorization followed by specific identification. We speculate that the brain might use this type of coding for other biologically important categories as well, as some brain-damaged patients are impaired only with regard to particular categories other than faces, such as food, objects or tools^{25,26}. □

Methods

Task procedures. Two monkeys (*Macaca fuscata*) were trained to perform a fixation task. The monkey initiated a trial by pressing a button to bring out a target red spot ($0.3^\circ \times 0.3^\circ$) in the centre of a colour monitor screen located 48 cm in front of the eyes. After the red spot had been displayed for 600 ms, a 250-ms long blank period commenced and then a test stimulus was presented for 350 ms. Finally, the red spot re-appeared and was replaced by the blue spot after a random period (500–1,250 ms). Within the last period, the monkey was required to release the button and was rewarded. Using a PC-based CCD camera system, the eye movements were monitored, and if the monkey's eye deviated more than 1° from the centre of the screen, the trial was automatically terminated.

Recording and histologic analysis. After training, each monkey was implanted with chronic recording chambers on the skull under anaesthesia. A parylene-coated tungsten microelectrode was inserted vertically through a guide tube fixed to the grid. Extracellular action potentials of single neurons were recorded by the electrode, discriminated on-line, and converted into pulses. Timing of spikes of a neuron and of task-related events were stored on a PC with a time resolution of 1 ms. After the recording sessions, the histological localization of recorded neurons in the first monkey was identified by analysing cerebrum sections. The areas recorded in the other monkey were examined by both X-ray and magnetic resonance images.

Data analysis. The stored data were analysed off-line on a workstation (SiliconGraphics, INDIGO2) using MATLAB. To determine whether recorded neurons responded to facial stimuli, averaged peristimulus time histograms of all test stimuli were outlined for each neuron (bin width, 10 ms). If a neuron showed significant responses to any facial stimuli (threshold criterion, the mean + 2 times the s.d. of the responses within a 140-ms period before the stimulus onset ($P < 0.05$)), it was considered a 'face-responsive' neuron. Among these neurons, 86 neurons tested with more than four trials per stimulus (the median number of trials was 199) were analysed as follows.

The facial information was divided into one global and four fine categories, and each predictable information associated with an occurrence of neuronal responses ($I(S; R)$) was quantified as the decrease in entropy of the stimulus occurrence ($H(S)$):

$$I(S; R) = H(S) - H(S|R) = \sum_s -p(s) \log p(s) - \left\langle \sum_s -p(s|r) \log p(s|r) \right\rangle_r \quad (1)$$

Where S is the set of stimuli s , R is the set of signals r (the neuronal responses, that is, spike counts), $p(s|r)$ is the conditional probability of stimulus s given an observed spike count r , and $p(s)$ is the *a priori* probability of stimulus s . The brackets indicate an average of the signal distribution $p(r)$. We used a simple

procedure to evaluate 'significance' of information with the χ^2 test¹³. The statistical hypothesis of the χ^2 test stated that ' $p(s|r)$ was equal to $p(s)$ '. The information was considered 'significant' when the corresponding χ^2 test yielded $P < 0.05$ ($= \alpha$). If n responses (r_i , $i = 1, 2, \dots, n$) existed, then $P < \alpha/n$, using the Bonferroni correction. To execute the χ^2 test, if the *a priori* occurrence of s for a given r_i was less than 5, r_i was merged with r_{i-1} . The value of n (equal to the number of bins, B) in the analysis ranged from 1 to 7 for the global category, and from 1 to 4 for the fine category. In estimating the transmitted information with a small number of trials, the information is biased upward. We corrected the information estimation by subtracting the first-order correction term (C_1) from the value calculated using equation (1), as C_1 represents almost all the error due to limited sampling²⁷:

$$C_1 = \frac{1}{2N \ln 2} \{ \sum_s \bar{B}_s - B - (S - 1) \} \quad (\bar{B}_s \text{ denotes the number of non-zero response bins for the trials with stimulus } s).$$

Thus, $I_c = I(S; R) - C_1$. The performance of this subtraction has been shown to be accurate²⁸. After the correction, no correlation was observed between the trial numbers and the transmitted information in our results. To examine the time course of the information, the response was evaluated using 50-ms sliding windows. The middle of the window was moved in 8-ms steps beginning 5 ms after the stimulus onset, up to 509 ms. The information latency was measured from the stimulus onset to the first 'significant' window centre. To directly compare the latency of information with that of neuronal responses, the response histogram (beige histogram in Fig. 2a) was constructed by plotting the averaged number of spikes across all trials to all stimuli using 50-ms sliding windows, with the identical time sequence for the information analysis. The response latency was measured from the stimulus onset to the window centre when the response first exceeded a threshold criterion (mean + 2 times s.d. of the responses within 154-ms period before the stimulus onset ($P < 0.05$)). As latencies were defined as the time from stimulus onset to the middle of the 50-ms window, the response and information latencies in this study are shorter than those in published data^{4,8,9}.

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- Gross, C. G., Rocha-Miranda, C. E. & Bender, D. E. Visual properties of neurons in inferotemporal cortex of the macaque. *J. Neurophysiol.* **35**, 96–111 (1972).
- Bruce, C., Desimone, R. & Gross, C. G. Visual properties of neurons in a polysensory area in superior temporal sulcus of the macaque. *J. Neurophysiol.* **46**, 369–384 (1981).
- Perrett, D. I. et al. Neurones responsive to faces in the temporal cortex: Studies of functional organization, sensitivity to identity and relation to perception. *Hum. Neurobiol.* **3**, 197–208 (1984).
- Baylis, G. C., Rolls, E. T. & Leonard, C. M. Functional subdivisions of the temporal lobe neocortex. *J. Neurosci.* **7**, 330–342 (1987).
- Hasselmo, M. E., Rolls, E. T. & Baylis, G. C. The role of expression and identity in the face-selective responses of neurons in the temporal visual cortex of the monkey. *Behav. Brain Res.* **32**, 203–218 (1989).
- Yamane, S., Kaji, S. & Kawano, K. What facial features activate face neurons in the inferotemporal cortex of the monkey? *Exp. Brain Res.* **73**, 209–214 (1988).
- Young, M. P. & Yamane, S. Sparse population coding of faces in the inferotemporal cortex. *Science* **256**, 1327–1331 (1992).
- Mikami, A., Nakamura, K. & Kubota, K. Neuronal responses to photographs in the superior temporal sulcus of the rhesus monkey. *Behav. Brain Res.* **60**, 1–13 (1994).
- Nakamura, K., Matsumoto, K., Mikami, A. & Kubota, K. Visual response properties of single neurons in the temporal pole of behaving monkeys. *J. Neurophysiol.* **71**, 1206–1221 (1994).
- Oram, M. W. & Perrett, D. I. Time course of neural responses discriminating different views of the face and head. *J. Neurophysiol.* **68**, 70–84 (1992).
- Oram, M. W. & Perrett, D. I. Integration of form and motion in the anterior superior temporal polysensory area (STPa) of the macaque monkey. *J. Neurophysiol.* **76**, 109–129 (1996).
- Tovee, M. J., Rolls, E. T., Treves, A. & Bellis, R. P. Information encoding and the responses of single neurons in the primate temporal visual cortex. *J. Neurophysiol.* **70**, 640–654 (1993).
- Kitazawa, S., Kimura, T. & Yin, P. B. Cerebellar complex spikes encode both destinations and errors in arm movements. *Nature* **392**, 494–497 (1998).
- Sugase, Y., Yamane, S., Kawano, K. & Ueno, S. Neurons in the macaque temporal cortex convey multiple information about monkey and human faces with expression. *Soc. Neurosci. Abs.* **24**, 899 (1998).
- Heller, J., Hertz, J. A., Kjaer, T. W. & Richmond, B. J. Information flow and temporal coding in primate pattern vision. *J. Comput. Neurosci.* **2**, 175–193 (1995).
- Suzuki, W. A. & Amaral, D. G. Perirhinal and parahippocampal cortices of the macaque monkey: Cortical afferents. *J. Comp. Neurol.* **350**, 497–533 (1994).
- Lavenex, P., Suzuki, W. A. & Amaral, D. G. Projections from the perirhinal and parahippocampal cortices to the neocortex in macaque monkeys (*Macaca fascicularis*). *Soc. Neurosci. Abs.* **24**, 1905 (1998).
- Seltzer, B. & Pandya, D. N. Afferent cortical connections and architectonics of the superior temporal sulcus and surrounding cortex in the rhesus monkey. *Brain Res.* **149**, 1–24 (1978).
- Webster, M. J., Bachevalier, J. & Ungerleider, L. G. Connections of inferior temporal areas TEO and TE with parietal and frontal cortex in macaque monkeys. *Cereb. Cortex* **4**, 470–483 (1994).
- Amaral, D. G. & Price, J. L. Amygdalo-cortical projections in the monkey (*Macaca fascicularis*). *J. Comp. Neurol.* **230**, 465–496 (1984).
- Nakamura, K., Mikami, A. & Kubota, K. Activity of single neurons in the monkey amygdala during performance of a visual discrimination task. *J. Neurophysiol.* **67**, 1447–1463 (1992).
- Amari, S. Neural theory of association and concept-formation. *Biol. Cybern.* **26**, 175–185 (1977).

23. Ellis, H. D. in *The Neuropsychology of Face Perception and Facial Expression* (ed. Bruyer, B.) 1–27 (Lawrence Erlbaum, London, 1986).
24. Hecaen, H. in *Interhemispheric Relations and Cerebral Dominance* (ed. Moutcastle, V. B.) 215–243 (John Hopkins, Baltimore, 1962).
25. McCarthy, R. A. & Warrington, E. K. Evidence for modality-specific meaning systems in the brain. *Nature* **334**, 428–430 (1988).
26. Damasio, H., Grabowski, T. J., Tranel, D., Hichwa, R. D. & Damasio, A. R. A neural basis for lexical retrieval. *Nature* **380**, 499–505 (1996).
27. Panzeri, S. & Treves, A. Analytical estimates of limited sampling biases in different information measures. *Network* **7**, 87–107 (1996).
28. Golomb, D., Hertz, J., Panzeri, S., Treves, A. & Richmond, B. How well can we estimate the information carried in neuronal responses from limited samples? *Neural Comput.* **9**, 649–665 (1997).

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Caudal is the Hox gene that specifies the most posterior *Drosophila* segment

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The homeobox gene *caudal* (*cad*) has a maternal embryonic function that establishes the antero–posterior body axis of *Drosophila*^{1,2}. It also has a conserved^{3,4} late embryonic and imaginal function¹ related to the development of the posterior body region. Here we report the developmental role of *cad* in adult *Drosophila*. It is required for the normal development of the analia structures, which derive from the most posterior body segment. In the absence of *cad* function, the analia develop like the immediately anterior segment (male genitalia), following the transformation rule of the canonical Hox genes⁵. We also show that *cad* can induce ectopic analia development if expressed in the head or wing. We propose that *cad* is the Hox gene that determines the development of the fly's most posterior segment. *cad* acts in combination with the Hedgehog (Hh) pathway⁶ to specify the

different components of the analia: the activities of *cad* and of the Hh pathway induce *Distal-less* expression that, together with *cad*, promote external analia development. In the absence of the Hh pathway, *cad* induces internal analia development, probably by activating the *brachyenteron* and *even-skipped* genes.

Segment identity along the antero–posterior body axis of *Drosophila* is determined by the genes of the *Hox* complex⁵, but there are two regions that develop normally in the absence of *Hox* gene activity: the anterior head region and the most posterior body segment, which includes the anal structures of larvae and adults⁵. A candidate for the specification of the latter is the homeobox gene *caudal* (*cad*), which is expressed in the posterior body region^{1,7}. In adult flies *cad* expression is restricted to the cells that form the analia^{8,9} (Fig. 1c), indicating a developmental function for *cad* in the analia.

The analia of *Drosophila* derive from the last abdominal segment (A10), which is fused with the primordia of segments A9 (that differentiates male genitalia) and A8 (that differentiates female genitalia), to form the genital disc¹⁰. Transplantation experiments¹¹ subdivided the anal primordium into two subprimordia corresponding to the precursor cells of external (anal plates) and internal (hindgut) analia. We find (Fig. 1a–d) that this subdivision is reflected in the expression of the developmental genes *Distal-less* (*Dll*), *even-skipped* (*eve*) and *brachyenteron* (*byn*)^{12–14}. Whereas *cad* expression covers the entire analia^{8,9}, *Dll* is restricted to the anal plates (Fig. 1a, c, d), and *byn* (Fig. 1b, d) and *eve* are both expressed

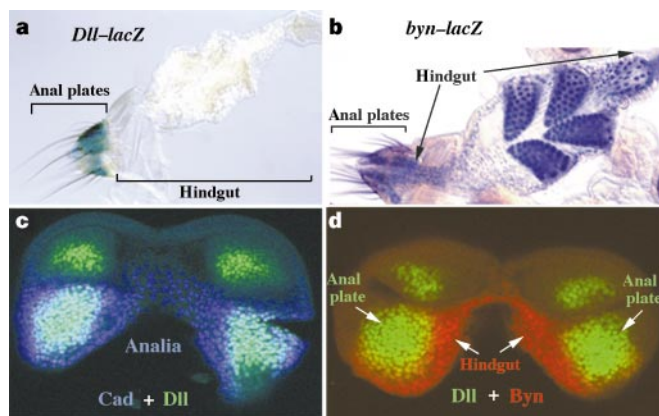


Figure 1 Analia subdomains in adult derivatives and imaginal discs. **a**, X-gal staining of the adult analia of *Dll-lacZ* flies. *LacZ* activity (blue) is restricted to the anal plates. **b**, X-gal staining of a *byn-lacZ* line²⁶ showing *LacZ* activity restricted to the hindgut. **c**, Genital disc stained for Cad (blue) and Dll (green) proteins. The Dll subdomain (whitish) occupies part of the Cad domain; the central region does not contain Dll protein. **d**, Genital disc stained for Dll (green) and Byn (red) proteins. The Dll (anal plates) and Byn (hindgut) subdomains show little or no overlapping and together cover most of the Cad domain (compare with **c**).

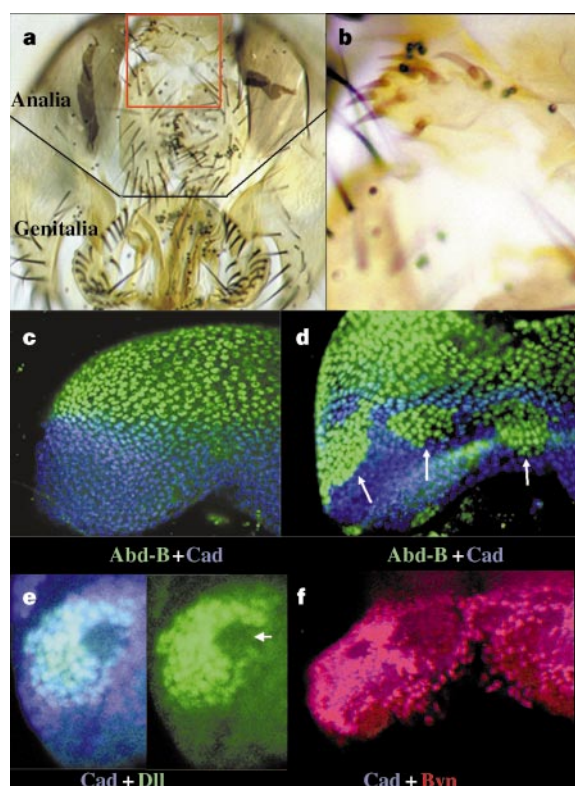


Figure 2 Homeotic transformations of *cad*⁻ clones. **a**, Male terminalia with a clone (within the red square) transforming analia into genitalia. **b**, The same clone at higher magnification showing characteristic male genitalia bristles (marked with yellow). These bristles are like those in the genitalia in **a**. **c**, Wild-type disc stained for Cad (blue) and Abd-B (green) proteins. The two products occupy distinct regions of the disc. **d**, *cad*⁻ clones (arrows), marked by the loss of Cad and showing ectopic Abd-B expression. **e**, Disc stained for Cad (blue) and Dll (green) containing a *cad*⁻ clone. The green channel is shown on the right to show the loss of *Dll* expression (arrow). **f**, Several *cad*⁻ clones in a disc stained for Cad (blue) and Byn (red) showing loss of *byn* expression.

only in the hindgut. *Dll* function is required for anal plate development (see below) and, as there is evidence that *byn* function is necessary for the larval hindgut¹⁵, we assume that it performs a similar function in the adult. *eve* mutant embryos have normal posterior regions¹⁶, indicating that, in spite of its expression, *eve* has no function there (see also below).

We have studied the requirements for *cad* function in the formation of the analia and for *Dll*, *eve* and *byn* expression by inducing clones of cells homozygous for the null *cad*² allele. In the absence of *cad* activity, the analia (A10) are homeotically transformed into the immediately anterior segment, male genitalia (A9). In males, *cad*[−] clones show a transformation into male genitalia, as indicated by characteristic pattern elements (Fig. 2a, b). In females, the transformation into male genitalia by *cad*[−] clones is not expected to be observed because differentiation of the male genital primordium is repressed¹⁰. Accordingly, we fail to detect these clones, but in an experiment in females (see Methods) in which *cad*[−] clones in twin with *cad*[−] were marked, *cad*⁺ clones frequently appeared close to undifferentiated patches, which we interpret as *cad*[−] clones by examining the expression of *Abdominal-B* (*Abd-B*), which is active in the primordium of male genitalia but not in the analia¹⁷ (Fig. 2c). *cad*[−] clones show *Abd-B* expression in male and female discs (Fig. 2d). We also examined *byn*, *eve* and *Dll* expression in *cad*[−] clones (Fig. 2e, f): *byn* and *eve* are not expressed, even in clones induced during the third larval period. *Dll* activity is lost in *cad*[−] clones induced before 72 h of development; late clones do not affect *Dll* expression. As *Dll* is autoregulatory¹⁸, it is possible that it needs

cad only for its initial activation. These results indicate that *cad* acts upstream of *Dll*, *eve* and *byn*, and that *cad* function may be prerequisite for their activation.

We tested the developmental potential of *cad* using the Gal4/UAS method¹⁹ to force *cad* function out of its normal context. As a rule we used the MS-248 and the *apterous*–Gal4 driver lines, which confer expression in the head and wing and haltere discs⁸, respectively. MS-248/UAS–*cad* flies show ectopic analia structures, anal plates and hindgut, in the head capsule (Fig. 3a, b, d–f), showing that the Cad product can induce full analia development. In the wing of *ap*–Gal4/UAS–*cad* flies anal plates, but not hindgut, are found (Fig. 3h, i). No anal structures appear in the halteres of these flies.

The transformation induced by Cad is reflected in the eye–antenna and wing discs by ectopic expression of *Dll*, *byn* and *eve*. In MS-248/UAS–*cad* eye–antennal discs, Cad induces *Dll*, *byn* (Fig. 3c, g) and *eve* activity (not shown) in distinct non-overlapping domains. In contrast, in the wing disc of *ap*–Gal4/UAS–*cad* flies we find ectopic *Dll* but not *byn* or *eve* expression (Fig. 3j), and in the haltere disc neither *Dll* nor *eve* or *byn* are ectopically expressed. These observations indicate that the response to the Cad product is modulated by local factors.

We show above that Cad activates *Dll*, but in the leg disc *Dll* expression requires the Hh-dependent Wg and Dpp signals²⁰. As in the analia primordium *hh*, *wg* and *dpp* expression resembles that in the leg disc (Fig. 4a–d), we tested whether Hh/Wg/Dpp signalling is required for *Dll* expression. Mutant larvae for *hh*^{ts-2}, *wg*^{ts} and

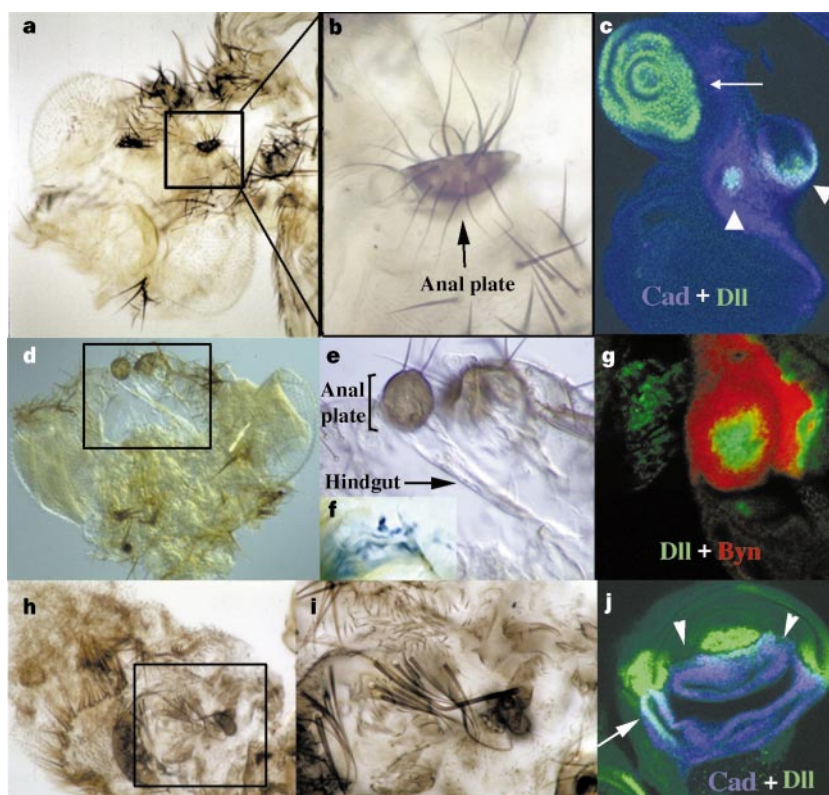


Figure 3 Ectopic *cad* expression. **a**, Head and thorax of an MS-248 UAS–*cad* fly. Anal plate structures (square) appear in the head, shown at higher magnification in **b**, to point out characteristic and plate bristles (arrow). **c**, An eye–antennal disc of the same genotype as **a** and **b**, stained for Cad and Dll. The bigger patch of Dll protein (arrow) corresponds to its normal expression in the antenna, but there are two areas of *Dll* expression (arrowheads) within the region containing Cad. **d**, **e**, Head of the same genotype with (square) anal plates associated with a non-cuticular area resembling hindgut, which is amplified in **e**, **f**. Its hindgut nature can be demonstrated by introducing in this genotype the *byn*–*lacZ* chromosome²⁶

(Fig. 1b). X-gal staining reveals *lacZ* activity in these cells. **g**, Double staining for Dll and Byn of another eye–antennal disc of the same genotype to show that Cad induces the expression of *Dll* and *byn* in non-overlapping domains. **h**, Wing of an *ap*–Gal4/UAS–*cad* female with anal plates (square). **i**, Higher magnification of the region marked in **h** to show the characteristic bristles of the female and anal plates. **j**, Wing disc of the same genotype stained for Dll and Cad. In comparison with the wild-type²⁸, it shows increased *Dll* expression in the proximal regions (arrow), and loss of *Dll* expression in the distal region (arrowheads).

dpp^{D12}/dpp^{D14}, which strongly reduce or eliminate the corresponding function²⁰, were used, and their genital discs were examined for *Dll*, *eve* and *byn* expression. No *Dll* activity was detected in any of the three combinations. Instead, a large majority of cells show activity for *eve* and *byn* (Fig. 4f–i), indicating that hindgut development is unaffected. Thus the *Dll* subdomain is dependent on the activity of the Hh pathway but the Byn/Eve subdomain is not. Moreover, the finding that loss of either *wg* or *dpp* activity abolishes *Dll* expression, just like the loss of *hh*, indicates that Hh may act indirectly through Wg and Dpp.

These results indicate a subdivision of the Cad domain in which the zone within the effective range of the Wg and Dpp signals becomes the *Dll* subdomain, whereas the Byn/Eve subdomain is outside their range. If this subdivision is based on the diffusion of Wg and Dpp, the boundary between the two subdomains is not expected to be a cell-lineage boundary. We induced clones marked with *lacZ* activity in which either the *Dll* or the Byn/Eve subdomain were labelled with the corresponding antibody. Even clones induced during the third larval period frequently crossed the border between the *Dll* and Byn/Eve subdomains (Fig. 4e).

The preceding results indicate a model of regionalization of the analia: the Cad product alone specifies hindgut development, probably by activating *byn*, *eve* and other target genes. In the region within the range of Wg and Dpp, Cad activates *Dll*, and their combined activities specify anal plates. Hindgut-specific Cad target genes such as *byn* and *eve* are repressed in this primordium.

One prediction of this model is that activation of the Hh pathway in the hindgut should produce a transformation into anal plates. We have tested this by forcing high levels of *Cubitus interruptus* (*Ci*) in the analia primordium, as expression of *Ci* at those levels is sufficient to activate Hh target genes in the absence of *hh* activity²¹. We find that overexpression of *Ci* in *cad-gal4/UAS-Ci* flies produces very large anal plates (67 average number of bristles versus wildtype (37), Fig. 5a, b), presumably owing to the transformation of the hindgut (Fig. 5c). Correspondingly, the analia primordium shows an expansion of the *Dll* subdomain at the expense of the Byn/Eve subdomain (Fig. 5d).

A complementary prediction is that blocking Hh/Wg/Dpp signalling should prevent *Dll* activity and transform the anal plates into hindgut. We induced clones of cells deficient for *smoothed* (*smo*) function, which lack an essential element of the Hh transduction pathway²². As cuticle markers are not scorable in hindgut tissue, we labelled the twin of the *smo*[−] clone using the marker *crinkled* (*ck*). Small, late-induced clones had little effect, but those initiated before 72 h appeared frequently (16 out of 21) to be associated with pieces of non-cuticular tissue (Fig. 5e) that we interpret as hindgut. In the *Dll* subdomain these clones lose *Dll* (not shown) and acquire *byn* expression (Fig. 5f), although not all the cells in *smo*[−] clones show these effects. This non-autonomy supports the idea that the formation of the two subdomains is mediated by Wg and Dpp.

To test if the repression of *byn* is mediated by *Dll*, we have examined genital discs of *Dll* hypomorphic mutants and clones of cells mutant for the null *Dll^{Δ1}* allele. The mutant discs show diminished *Dll* expression and an expansion of *byn* expression. *Dll*[−] clones were unable to form anal plates (Fig. 5g, see Methods for details), but they proliferate normally and many of them show *byn* and *eve* expression (Fig. 5h), especially those close to the Byn/Eve subdomain. Thus, *Dll* (and possibly other genes) functions as a negative regulator of *byn* and *eve*, suppressing the activating function of Cad on these genes.

We have identified *caudal* as the homeobox gene that determines the identity of the most posterior (A10) segment of *Drosophila*. Its function conforms to the rules of the canonical Hox genes: (1) *cad* is exclusively expressed in the analia, a segmental derivative in which none of the Hox-complex genes is active. This domain abuts the expression domain of *Abd-B*, the most posterior Hox gene; (2) in the absence of *cad* function, analia structures are transformed into those corresponding to the immediately anterior segment, male genitalia; (3) *cad* downregulates the expression of *Abd-B*; (4) ectopic *cad* expression can induce analia development in inappropriate places, such as head or wing; and (5) *cad* is required for, and induces, the activity of the downstream genes like *Dll*, *byn* and *eve* that are involved in the specification of various components of the analia. Thus, *cad* behaves as expected for a hypothetical Hox-

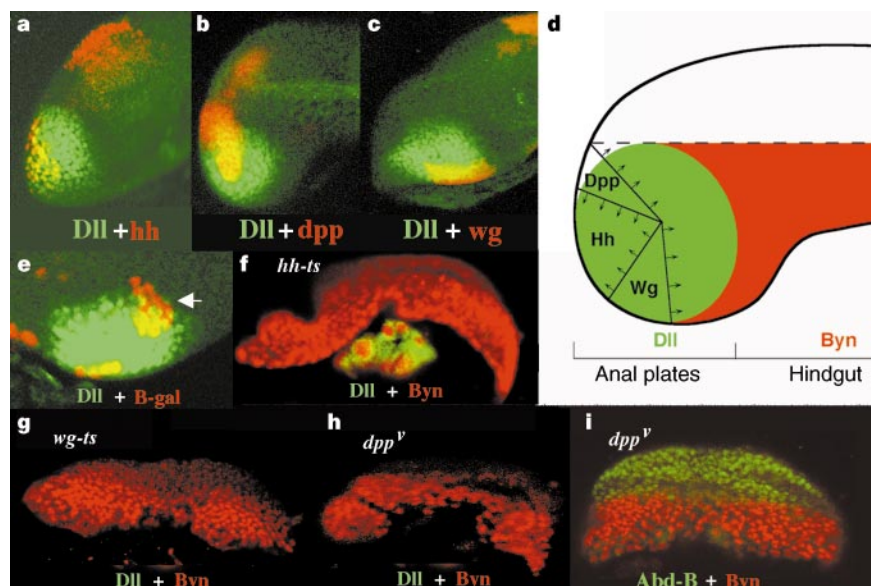


Figure 4 The Hh pathway in the analia primordium. **a, b, c**, Double staining of *Dll* and *hh*, *dpp* and *wg*, respectively. These genes are expressed in the lateral region of the primordium and their disposition resembles that of the leg disc²⁰. **d**, Diagram of expression patterns shown in **a–c**. The region expressing *byn* is opposite to the source of Wg and Dpp. **e**, Clones of *lacZ*-expressing cells induced during the third larval period. These clones can cross (arrow) the boundary

between the *Dll* (green) and Byn/Eve subdomains. **f, g, h**, *hh^{ts2}*, *wg^{IL114}* and *dpp^v* (*dpp^{D12}/dpp^{D14}*) mutant discs stained for *Dll* and Byn. No *Dll* expression is detected. **i**, *dpp^v* disc stained with Byn and *abd-B* (which marks female and male genitalia) showing that what is left of the analia primordium is almost entirely covered by *byn* expression.

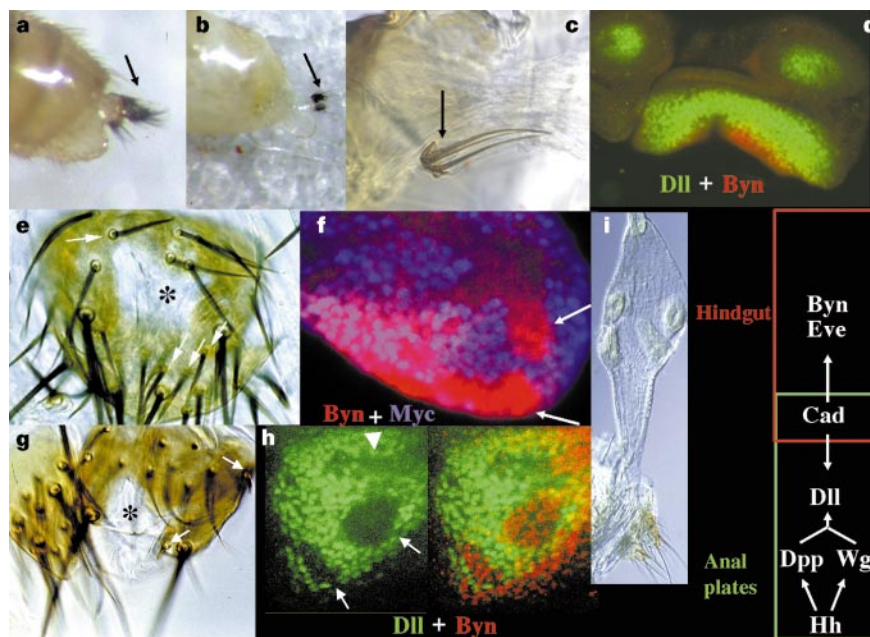


Figure 5 Interactions between *cad* and the Hh pathway. **a**, Fly of genotype *yw*; *cad-Gal4 UAS-ci UAS-y* showing enlarged anal plates, about twice the normal size (compare with **b**). The *UAS-y* construct⁶ marks the anal plates with *y*⁺. **b**, Fly of genotype *yw*; *cad-Gal4 UAS-y* at the same magnification to show the normal size of anal plates, also marked with *y*⁺. **c**, Example of anal plate bristles that occur within the hindgut of *cad-Gal4 UAS-ci* flies. **d**, Genital disc of *cad-Gal4 UAS-ci* genotype showing the enlargement of the Dll domain that occupies most of the Byn/Eve domain. **e**, *smo*⁻ clone marked with *crinkled* (arrows). Nearby there is a non-cuticular area (asterisk) that we interpreted as a twin *smo*⁻ clone differentiating hindgut. **f**, *smo*⁻ clones (arrows) marked with the loss of Myc protein and showing gain of *byn* expression. The gain of *byn* expression is only in part of the upper clone. **g**, *Dll*⁺ clone marked with *pwn* (arrows). Nearby there is a zone

of defective cuticle (asterisk) that we interpret as being formed by the *Dll*⁻ cells. **h**, *Dll*⁻ clones stained for Dll and Byn. Two clones (arrows) show *byn* expression, but one in the upper part (arrowhead) does not. **i**, A combinatorial model to specify the development of the components of the analia. The Cad product is present in the entire primordium. In the anal plate subprimordium (green box) there is *hh* activity that induces *Wg* and *Dpp* signals. These and the Cad product activate *Dll* and possibly other genes. Cad and Dll activities are both necessary for anal plate development. In the hindgut subprimordium (red box) the activity of Cad activates *byn*, *eve* and possibly other specific genes. It is not clear whether *cad* has a morphogenetic function independent of *byn* and *eve* as the elimination of *cad* function always results in the loss of *byn* and *eve* expression.

complex gene located 5' of *Abd-B* that would specify a body domain posterior to that of *Abd-B*.

cad homologues in nematodes and chordates are also expressed in the posterior body regions^{3,4}. Moreover, mice mutant for *Cdx2* show abnormal development of the tail and posterior intestine²³, indicating that *cad* function is conserved. *cad* may have been an original member of the Hox cluster that split during evolution. However, *cad* homologues are not physically linked to the Hox cluster in any animal group, so if splitting occurred it must have been very early in the evolution of the cluster. It has been suggested⁴ that *cad* is a member of a 'ParaHox' complex descending from a 'ProtoHox' gene cluster common to the Hox and ParaHox complexes. *cad* and *Abd-B* would be the paralogous most 5' genes in the ParaHox and Hox complexes, respectively⁴. A difficulty with this view is that, in *Drosophila*, *Abd-B* and *cad* have different and non-overlapping expression domains, which would not be expected if they were paralogous genes. We note, however, that the domain of the homeobox gene *eve* is partially coincident with the Cad domain and that *eve* homologues are located 5' of *Abd-B* in the vertebrate Hox complex⁵ and in cnidarians²⁴, indicating that *eve* was a member of the original cluster. We propose that *eve* and *cad* are paralogous members of the Hox and ParaHox complexes, respectively. They are both active in the most posterior body region, as dictated by the colinearity rule⁵, but in *Drosophila* the original function of *eve* may have been over-ridden by *cad*. This is consistent with the lack of effect of *eve* mutations in the posterior regions¹⁶.

Our results also suggest a combinatorial model for the specification of the two analia components, anal plates and hindgut (Fig. 5i): *cad* by itself specifies hindgut development, but in combination

with *Dll* and possibly other *Wg* and *Dpp* response genes, they repress hindgut and induce anal plate development. The critical event appears to be the formation within the Cad domain of two morphogenetic fields, the Dll and Byn/Eve subdomains. In the zone close to their origin, *Wg* and *Dpp* trigger *Dll* and other response genes, but in the cells located medially, *Wg* and *Dpp* do not reach sufficient concentrations to activate these genes. Then *byn* activity and subsequently hindgut development would follow by default. In this model the subdivision of the analia into anal plates and hindgut would be a consequence of the effective range of the *Wg* and *Dpp* signals. □

Methods

Fly stocks, crosses and production of marked clones. The *hh-lacZ*, *dpp-lacZ*, *wg-lacZ* and *byn-lacZ* stocks were used to describe the corresponding expression patterns in the analia^{25,26}. We studied loss of *cad* function using the *cad*¹ and null¹ *cad*² alleles. Mutant *cad*² clones were generated using the FLP/FRT method by heat-shocking at 37°C (1–2 h) larvae of genotype *cad*² FRT40/*Dpse*¹⁹, *y*⁺ *DpW*⁺ FRT40 to recover clones marked with *yellow*, and of genotype *ywf* FLP122; *cad*² FRT40/*ck* *f*⁺ FRT40 to mark the twin *cad*⁺ clone with *crinkled* (*ck*). In the discs *cad*² clones were identified by the lack of Cad protein. To study the cell lineage of the Dll and Byn/Eve subdomains, we induced *lacZ*-expressing clones using an *actin5C > Draf > lacZ* construct⁶ with a short heat shock of 5–8 min, to obtain few clones per disc. To induce *smo*³ clones in adults, larvae of genotype *ywf* FLP122; *smo*³ FRT40/*ck* *f*⁺ FRT40 were heat shocked either during the second (48–72 h of development) or the third (72–96 h) larval period. Twin *smo*⁺ clones are marked with *ck*. *smo*³ clones in discs were induced in *ywf* FLP122; *smo*³ FRT39/*hs-myc* FRT39 larvae and detected by the absence of Myc protein. For clones lacking *Dll* activity we used the *Dll*^{SAI}

null mutation. Two different genotypes were used to mark mutant clones: *yw FLP122; FRT42D Dll^{SA1}/FRT42D Dp(y⁺)44B* or *yw FLP122; FRT42D Dll^{SA1}/FRT42D pwn*. In the first genotype, *Dll* heat-shock-induced clones were labelled with the cuticle marker *y*. As a possible transformation into hindgut could not be scored with this system, in the second genotype the marker *pwn* labels the *Dll*⁺ clones twins of the *Dll*^{SA1} clones. For *Dll* clones²⁷ in the genital disc, larvae of genotype *yw FLP122; FRT42D Dll^{SA1}/FRT42D arm-lacZ* were given 1-h heat shocks and *Dll*^{SA1} clones were marked by the loss of either *Dll* protein or *lacZ* activity. Partial loss of *Dll* function was studied in trans combinations of the *Dll*³, *Dll*^{MP} and *Dll-Gal4* alleles⁸. We studied the requirements of the anialia for *hh*, *wg* and *dpp* function using the *hh*^{ts2}, *wg*¹¹¹⁴, *dpp*^{d12} and *dpp*^{d14} alleles as described²⁰. To activate the Hh pathway²¹ in the entire anialia primordium we generated *yw; cad-Gal4/UAS-ci; UAS-y* flies, in which the Ci product is expressed in the whole of the anialia primordium. The anal plates of these flies are marked by the expression of the *UAS-y* gene, which confers a dark colour.

Ectopic *cad* expression. We used the Gal4/UAS method¹⁹. A *UAS-cad* gene was constructed by inserting the *cad* complementation DNA (a gift from R. Rivera-Pomar) in the pUAS vector as described⁸. The *UAS-cad* construct is function, as it can rescue the mutant phenotype of the *cad-Gal4* insertions. The MS-248 (a gift from C. Parras) and *ap-Gal4*⁸ driver lines gave the best results. Flies of genotype *MS-248/UAS-cad* and *ap-Gal4/UAS-cad* were mounted and examined at the light microscope.

X-gal and antibody staining. X-gal staining of adult flies and imaginal discs was performed as described⁸. For adults it is important that they had emerged shortly before fixing. Imaginal discs were immunostained using the normal procedures for confocal microscopy. The specific antibodies used were rabbit anti-Byn (a gift from T. Kusch), rabbit anti-Cad (from G. Struhl), monoclonal anti-Dll (from S. Cohen and I. Duncan), rabbit anti-Eve (from M. Frusta) and monoclonal anti-Abd-B (from S. Celniker and A. Macias).

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- Macdonald, P. & Struhl, B. A molecular gradient in early *Drosophila* embryos and its role in specifying the body pattern. *Nature* **324**, 537–545 (1986).
- Mlodzik, M., Fjose, A. & Gehring, W. J. Isolation of *caudal*, a *Drosophila* homeobox-containing gene with maternal expression, whose transcription forms a concentration gradient at the pre-blastoderm stage. *EMBO J.* **4**, 2961–2969 (1985).
- Hunter, C. P. & Kenyon, C. Spatial and temporal controls target *pal-1* blastomere-specification activity to a single blastomere lineage in *C. elegans* embryos. *Cell* **87**, 217–226 (1997).
- Brooke, N. M., Garcia-Fernandez, J. & Holland, P. W. The ParaHox gene cluster is an evolutionary sister of the Hox gene cluster. *Nature* **392**, 920–922 (1998).
- McGinnis, W. & Krumlauf, R. Homeobox genes and axial patterning. *Cell* **68**, 283–302 (1992).
- Basler, K. & Struhl, G. Compartment boundaries and the control of *Drosophila* limb pattern by Hedgehog protein. *Nature* **368**, 208–214 (1994).
- Wu, L. H. & Lengyel, J. A. Role of *caudal* in the hindgut specification and gastrulation suggests homology between *Drosophila* amnioproctodeal invagination and vertebrate blastopore. *Development* **125**, 2433–2442 (1996).
- Calleja, M., Moreno, E., Pelaz, S. & Morata, G. Visualization of gene expression in living adult *Drosophila*. *Science* **274**, 252–255 (1996).
- Casares, F., Sanchez, L., Guerrero, I. & Sanchez-Herrero, E. The genital disc of *Drosophila melanogaster*. I. Segmental and compartmental organization. *Dev. Genes Evol.* **207**, 216–228 (1997).
- Nöthiger, R., Dübendorfer, A. & Epper, F. Gynandromorphs reveal two separate primordia for male and female genitalia in *Drosophila melanogaster*. *Wilhelm Roux Arch. Dev. Biol.* **181**, 367–373 (1977).
- Epper, F. Three-dimensional fate map of the female genital disc of *Drosophila melanogaster*. *Wilhelm Roux Arch. Dev. Biol.* **192**, 270–274 (1983).
- Cohen, S. M., Bröner, G., Kuntter, F., Jurgens, G. & Jackle, H. *Distal-less* encodes a homeodomain protein required for limb development in *Drosophila*. *Nature* **338**, 432–434 (1989).
- Frasch, M., Hoey, T., Rushlow, C., Doyle, H. & Levine, M. Characterization localization of the *even-skipped* protein of *Drosophila*. *EMBO J.* **6**, 749–759 (1987).
- Kispert, A., Herrmann, B. G., Leptin, M. & Reuter, R. Homologs of the mouse *Brachyury* gene are involved in the specification of posterior terminal structures in *Drosophila*, *Tribolium*, and *Locusta*. *Genes Dev.* **8**, 2137–2150 (1994).
- Singer, J. B., Harbecke, R., Kusch, T., Reuter, R. & Lengyel, J. A. *Drosophila brachyenteron* regulates gene activity and morphogenesis in the gut. *Development* **122**, 3703–3718 (1996).
- Nusslein-Volhard, C., Kluding, H. & Jurgens, G. Genes affecting the segmental subdivisions of the *Drosophila* embryo. *Cold Spring Harb. Symp. Quant. Biol.* **50**, 145–154 (1985).
- Sanchez-Herrero, E., Vernos, I., Marco, R. & Morata, G. Genetic organization of the *Drosophila* bithorax complex. *Nature* **313**, 108–113 (1985).
- Lecuit, T. & Cohen, S. M. Proximal–distal axis formation in the *Drosophila* leg. *Nature* **388**, 139–145 (1997).
- Brand, A. H. & Perrimon, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401–415 (1993).
- Diaz-Benjumea, F. J., Cohen, B. & Cohen, S. M. Cell interaction between compartments establishes the proximal–distal axis of *Drosophila* legs. *Nature* **372**, 175–179 (1994).
- Alexandre, C., Jacinto, A. & Ingham, P. W. Transcriptional activation of *hedgehog* target genes in *Drosophila* is mediated directly by the Cubitus interruptus protein, a member of the Gli family of zinc finger DNA-binding proteins. *Genes Dev.* **10**, 2003–2013 (1996).
- van den Heuvel, M. & Ingham, P. W. *smoothed* encodes a receptor-like serpentine protein required for *hedgehog* signalling. *Nature* **382**, 547–551 (1996).
- Chawengsaksophak, K., James, R., Hammond, V. E., Kontgen, F. & Beck, F. Homeosis and intestinal

- tumours in *Cdx2* mutant mice. *Nature* **396**, 84–87 (1997).
- Miller, D. J. & Miles, A. Homeobox genes and the zootype. *Nature* **365**, 215–216 (1993).
- Sánchez, L., Casares, F., Gorfinkel, N. & Guerrero, I. The genital disc of *Drosophila melanogaster*. II. Role of the genes *hedgehog*, *decapentaplegic* and *wingless*. *Dev. Genes Evol.* **207**, 229–241 (1997).
- Murakami, R. et al. *aprotous*, a locus that is necessary for the development of the proctodeum in *Drosophila* embryos, encodes a homolog of the vertebrate *Brachyury* gene. *Wilhelm Roux Arch. Dev. Biol.* **205**, 89–96 (1995).
- Gorfinkel, N., Morata, G. & Guerrero, I. The homeobox gene *Distal-less* induces ventral appendage development in *Drosophila*. *Genes Dev.* **11**, 2259–2271 (1997).
- Diaz-Benjumea, F. & Cohen, S. M. Serrate signals through Notch to establish a wingless-dependent organizer at the dorsal-ventral compartment boundary of the *Drosophila* wing. *Development* **121**, 4215–4225 (1995).

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Notch signalling controls pancreatic cell differentiation

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The pancreas contains both exocrine and endocrine cells, but the molecular mechanisms controlling the differentiation of these cell types are largely unknown. Despite their endodermal origin, pancreatic endocrine cells share several molecular characteristics with neurons^{1–5}, and, like neurons in the central nervous system^{6,7}, differentiating endocrine cells in the pancreas appear in a scattered fashion within a field of progenitor cells^{8,9}. This indicates that they may be generated by lateral specification through Notch signalling^{6,7}. Here, to test this idea, we analysed pancreas development in mice genetically altered at several steps in the Notch signalling pathway. Mice deficient for *Delta-like gene 1* (*Dll1*)¹⁰ or the intracellular mediator *RBP-Jk*¹¹ showed accelerated differentiation of pancreatic endocrine cells. A similar phenotype was observed in mice over-expressing *neurogenin 3* (*ngn 3*)¹² or the intracellular form of *Notch3* (ref. 13) (a repressor of Notch signalling). These data provide evidence that *ngn3* acts as pro-endocrine gene and that *Notch* signalling is critical for the decision between the endocrine and progenitor/exocrine fates in the developing pancreas.

In the Notch signalling system, ligand activation leads to intracellular cleavage of the Notch receptor. The activated intracellular domain of Notch receptors interacts with the DNA-binding protein RBP-Jk to activate expression of the negative basic helix–loop–helix (bHLH) *HES* genes, which, in turn, repress expression of downstream target genes^{6,7} including the *ngn* genes^{12,14–16}. Thus, high *ngn* expression is indicative of cells differentiating to a primary fate. We therefore first investigated *ngn3* expression in the developing pancreas in detail. Low, uniform expression of *ngn3* could be

detected in the dorsal pancreatic epithelium at the ~14-somite stage (embryonic day (E) 9) (Fig. 1a). By the 25-somite stage (E9.5), *ngn3* was expressed at high levels in a few cells of the dorsal pancreatic epithelium (Fig. 1b). *ngn3*⁺ cells in the ventral epithelium were first detected at E10.5 (Fig. 1b; and data not shown), in keeping with the delayed appearance of endocrine cells in the ventral compared with the dorsal pancreas^{4,5,8,9}. The relative number of *ngn3*⁺ cells increased between E9.5 and E15.5 (Fig. 1a–e), but then decreased such that, by E17.5 and in neonatal pancreas, there were only a few *ngn3*⁺ cells (Fig. 1f, g). Markers of differentiated pancreatic endocrine cells, such as the islet-specific LIM-homeodomain protein ISL1^{2,17} (Fig. 1a–g), the β -cell-specific homeodomain protein IPF1/PDX1 and the pancreatic hormones (not shown), were expressed in cells separate from *ngn3*⁺ cells, providing evidence that *ngn3*⁺ cells and post-mitotic endocrine cells represent distinct populations. This indicates that the *ngn3*⁺ cells may be endocrine precursor cells, and may correspond to the primary fate in a lateral specification model.

To test this idea, we used the *Ipfl/Pdx1* promoter¹⁸ to broaden the expression of *ngn3* to most pancreatic precursor cells in transgenic mice. E12.5 *Ipfl/Pdx1-*ngn3** transgenic embryos exhibited small and poorly branched pancreatic buds (Fig. 1h, o), consisting

primarily of differentiated endocrine cells, as indicated by the expression of ISL1 (Fig. 1i, p), glucagon (Fig. 1j, q), peptide YY (PYY)¹⁹ (Fig. 1k, r) and *NeuroD*^{20–22} (data not shown). The endocrine cells in the pancreatic buds of stage-matched control embryos were relatively fewer and more scattered (Fig. 1i–j). No carboxypeptidaseA⁺ cells were detected in the pancreatic buds of E12.5 *Ipfl/Pdx1-*ngn3** transgenic embryos (Fig. 1l, s). This block in pancreatic exocrine development also persisted at later stages, as indicated by the lack of both amylase⁺ (Fig. 1n, u) and carboxypeptidaseA⁺ (data not shown) cells in the bud of E15.5 transgenic embryos. Instead, endocrine cells expressing glucagon (Fig. 1m, t), PYY and ISL1 (data not shown) were observed. Thus, ectopic expression of *ngn3* in most of the early pancreatic precursor cells results in a massive, precocious differentiation of pancreatic progenitor cells into endocrine cells. The accelerated, premature differentiation of endocrine cells depletes the pool of pancreatic precursor cells, resulting in a shortage of cells that can proliferate, branch and differentiate into exocrine cells (Fig. 1n–u). Collectively, these data show that *ngn3* acts as a pro-endocrine gene during pancreatic development, a function analogous to that of *ngn* genes in neurogenesis^{14–16}.

These findings implicate the Notch signalling pathway in pancreatic cell-type specification. We next altered Notch signalling at

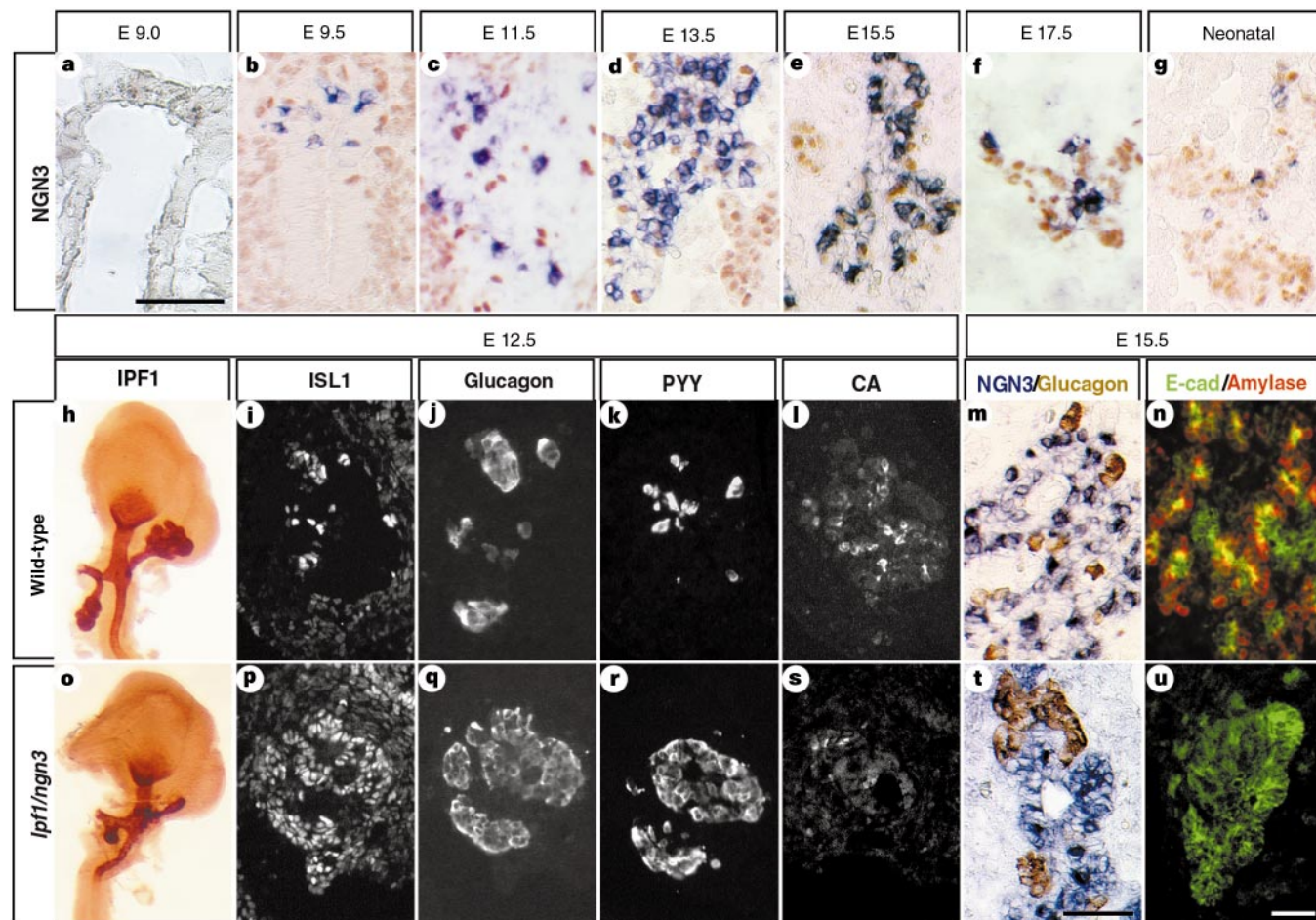


Figure 1 *ngn3* is expressed and functions as a pro-endocrine gene during pancreatic development. **a–g**, *ngn3* (dark blue) and ISL1 (brown) are expressed in separate sets of cells in the pancreas from E9.0 (**a**), E9.5 (**b**), E11.5 (**c**), E13.5 (**d**), E15.5 (**e**) and E17.5 (**f**) embryos and neonates (**g**). **h–u**, Accelerated pancreatic endocrine-cell differentiation in *Ipfl/Pdx1-*ngn3** transgenic mice. Whole-mount immunohistochemistry of the pancreatic region of E12.5 wild-type (**h**) and *Ipfl/Pdx1-*ngn3** transgenic (**o**) embryos using anti-IPF1/PDX1 antibodies shows impaired development of the pancreatic epithelium in transgenic embryos (**i–n**,

p–u). Accelerated endocrine cell differentiation occurs at the expense of exocrine cell differentiation in *Ipfl/Pdx1-*ngn3** transgenic pancreatic buds, as indicated by the presence of ISL1⁺ (**i**, **p**), glucagon⁺ (**j**, **q**, **m**, **t**) and PYY⁺ (**k**, **r**) pancreatic endocrine cells but lack of carboxypeptidaseA⁺ (**l**, **s**) and amylase⁺ (**n**, **u**) cells in E12.5 (**h–l**, **o–s**) and E15.5 (**m**, **n**, **t**, **u**) wild-type (**h–n**) and transgenic (**o–u**) embryos. Epithelial specific anti-E-cadherin (E-cad) antibodies (green) (**n**, **u**) were used to visualize the epithelium of E15.5 embryos and *ngn3*-expressing cells (blue) are shown by *in situ* hybridization and **m** and **t**. Scale bars, 0.05 mm.

different steps in the signalling pathway: at the ligand, receptor and intracellular mediator levels. *Notch1* (ref. 23) and *Notch2* (ref. 23), but not *Notch3* (ref. 23), were uniformly expressed in the epithelium at the pancreatic anterioposterior level of E9.5 wild-type embryos (Fig. 2a–c). *Dll1* (ref. 24), but not *Serrate1* (ref. 23), was also expressed at this stage, but only in the dorsal pancreatic epithelium (Fig. 2d, e). The restriction of *Dll1* expression to the dorsal epithelium of E9.5 embryos is consistent with the differentiation of endocrine cells in the dorsal, but not ventral, pancreatic bud at this stage. The *HES-1* gene²⁵ was, however, uniformly expressed in the E9.5 pancreatic epithelium (Fig. 2f), implying that other ligands may be expressed in the ventral pancreas at this stage. By E13.5, *Notch1*, *Notch2*, *Dll1* and *HES-1* were still expressed in the pancreatic epithelium (Fig. 2g, h, j, l) and both *Notch3* and *Serrate1* were also detected (Fig. 2i, k). These results show that components of the Notch pathway are expressed in the developing pancreas.

To assess the role of Notch signalling during pancreatic cell-type specification, we analysed mice deficient in *Dll1* (ref. 10) and *RBP-*

*Jk*¹¹, in which Notch signalling would be impaired at the ligand and intracellular mediator levels, respectively. *RBP-Jk* deficient mice die at around E8.5–9.5 (ref. 11). Nevertheless, analysis of E8.5–9.0 *RBP-Jk* mutant embryos revealed an apparently increased number of *ngn3*⁺ cells in the dorsal pancreatic epithelium (Fig. 3a, h; and data not shown). *Dll1* mutant mice are developmentally arrested between E10 and E12 (ref. 10) and thus only the early stages of pancreatic development can be investigated in these mice. *HES-1* expression was reduced in the dorsal epithelium of E9.5 *Dll1*^{−/−} embryos (Fig. 3b, i). Moreover, the total number of *ngn3*⁺ cells (55, 64 and 74 *ngn3*⁺ cells, respectively, in *n* = 3 embryos; Fig. 3j) was increased 3.5–4-fold in E9.5 *Dll1*^{−/−} embryos as compared with the few, scattered *ngn3*⁺ cells (11, 17 and 20 *ngn3*⁺ cells, respectively, in *n* = 3 embryos) in the dorsal pancreatic epithelium of *Dll1*^{+/+} embryos (Fig. 3c). By E10.5, differentiating endocrine cells were scattered among IPF1/PDX1⁺ progenitor cells within the protruding epithelial dorsal pancreatic bud (Fig. 3d, e, f, g). In E10.5 *Dll1* mutant mice, the pancreatic bud was decreased in size and instead a cap of differentiated endocrine cells expressing *NeuroD*/*BETA2*

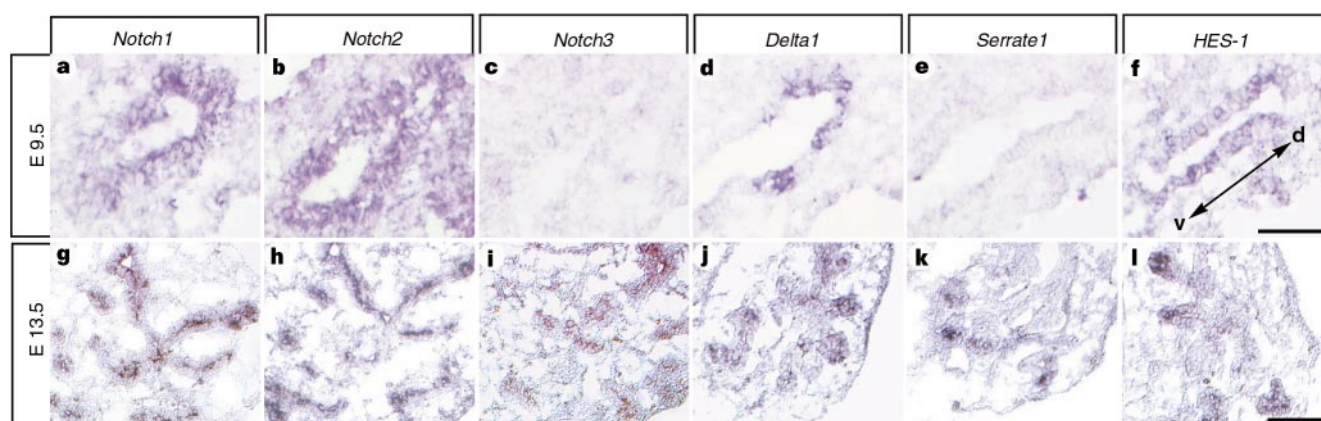


Figure 2 Notch signalling components are expressed in the developing pancreas. **a–l**, *In situ* hybridization analysis showing the expression in E9.5 (**a–f**) and E13.5 (**g–l**) pancreas of *Notch1* (**a, g**), *Notch2* (**b, h**), *Notch3* (**c, i**), *Delta1* (**d, j**), *Serrate1* (**e, k**) and *HES-1* (**f, l**). **a–f**, Dorsal–ventral axis is as shown in **f**. Scale bars in **a–f**, 0.05 mm; **g–l**, 0.1 mm.

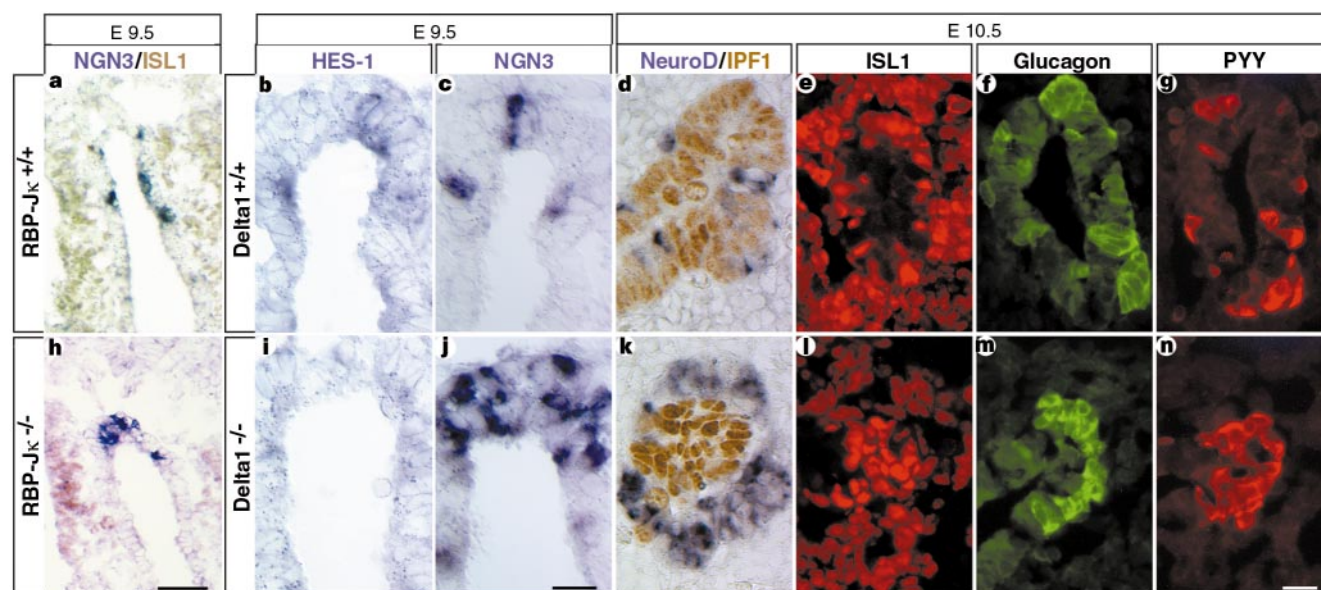


Figure 3 Lack of *RBP-Jk* and *Dll1* leads to precocious pancreatic endocrine differentiation. Analysis of E9.5 (**a–c, h–j**) and E10.5 (**d–g, k–n**) wild-type (**a–g**), *RBP-Jk*^{−/−} (**h**) or *Dll1*^{−/−} (**i–n**) mice using riboprobes corresponding to *ngn3* (**a, c, h, j**) *HES-1* (**b, i**) and *NeuroD* (blue) (**d, k**) and antibodies specific for IPF1/PDX1 (brown) (**d, k**), ISL1 (**a, e, h, l**), glucagon (**f, m**) and PYY (**g, n**) shows accelerated, premature differentiation of pancreatic endocrine cells in the mutant embryos. Scale bars in **a, h**, 0.05 mm; **b, c, i, j, d–g and k–n**, 0.02 mm.

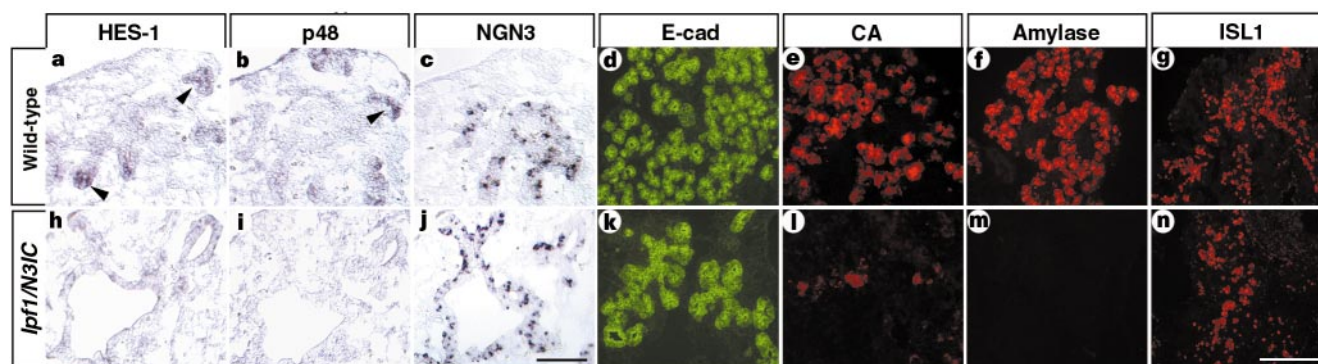


Figure 4 Repressed Notch signalling obstructs pancreatic epithelial branching and exocrine differentiation. **a–c, h–j**, The pancreatic epithelium is poorly branched, *HES-1* expression (**a, h**) is reduced, *p48* expression (**b, i**) is lost and *ngn3* (**c, j**) is expressed in cells throughout the pancreatic epithelium of E13.5 *Ipfl/Pdx1-N31C* transgenic (**h–j**) as compared with stage-matched wild-type (**a–c**) embryos. **d–n**, Immunohistochemical analysis of E15.5 wild-type (**d–g**) and *Ipfl/Pdx1-N31C* transgenic (**k–n**) pancreatic buds using antibodies specific for E-cadherin (**d, k**), carboxypeptidaseA (**e, l**), amylase (**f, m**) and ISL1 (**g, n**) show that, in the transgenic embryos, the pancreatic bud is decreased in size and poorly branched, and that exocrine, but not endocrine, cell differentiation is impaired. Scale bars in **a–c, h–j** and **d–g, k–n**, 0.1 mm.

(Fig. 3d, k), ISL1 (Fig. 3e, l), glucagon (Fig. 3f, m) and PYY (Fig. 3g, n) surrounded the IPF1/PDX1⁺ bud. The decrease in size of the pancreatic bud in *Dll1*^{−/−} embryos did not appear to be caused by increased apoptosis, as judged from TUNEL assays (data not shown). These results indicate that, in the absence of *Dll1*, epithelial, IPF1/PDX1⁺ progenitor cells within the pancreatic bud differentiate prematurely into endocrine cells, resulting in a depletion of cells within the pancreatic bud. These results provide evidence that lack of *Dll1* or *RBP-Jκ* (that is, impaired Notch signalling) leads to an accelerated differentiation of pancreatic precursor cells into endocrine cells.

N31C represses Notch 1-mediated up-regulation of *HES-1* expression *in vitro*²⁶. Thus, to experimentally perturb Notch signalling at the receptor level, we generated *Ipfl/Pdx1-N31C* transgenic mice. In wild-type E13.5 embryos, the *HES-1* (Figs 2l, 4a) and the exocrine transcription factor *p48* (refs 27, 28) (Fig. 4b) genes were highly expressed in the forming acini of the branching pancreatic epithelia, whereas developing endocrine cells, and thus *ngn3*⁺ cells, were found predominantly in the centre of the developing pancreatic bud (Fig. 4c). In contrast, the pancreatic epithelium of E13.5 *Ipfl/Pdx1-N31C* transgenic embryos was poorly branched and reduced in size (Fig. 4g, h). Furthermore, *p48* expression was absent (Fig. 4i), *HES-1* was expressed at a low level (Fig. 4h), and *ngn3*-expressing cells were found throughout the epithelium (Fig. 4j). The reduction in *HES-1* and the relative increase in *ngn3* expression are consistent with the function of *N31C* as a repressor²⁶. The concomitant loss of *p48* expression indicates that high *HES-1* expression may be required for *p48* expression. At E15.5, the pancreatic epithelia are normally highly branched and lobulated (Fig. 4d), and differentiated carboxypeptidaseA⁺ (Fig. 4e) and amylase⁺ (Fig. 4f) exocrine cells as well as ISL1⁺ (Fig. 4g) endocrine cells have appeared. In E15.5 *Ipfl/Pdx1-N31C* transgenic embryos, the pancreatic epithelium is poorly branched and the pancreatic buds are decreased in size (Fig. 4k and data not shown). In addition, very few carboxypeptidaseA⁺ (Fig. 4l) and no amylase⁺ (Fig. 4m) cells are observed, whereas differentiated endocrine cells are detected throughout the immature pancreatic epithelia, as judged by the expression of ISL1 (Fig. 4n) and hormones (data not shown). We conclude that *N31C* expression in the developing pancreas impairs pancreatic epithelial proliferation, morphogenesis and exocrine, but not endocrine, cell differentiation.

The results presented here provide new insight into cell lineage in the pancreas and show that the Notch signalling pathway is critical in pancreas development. Pancreatic cell fate is altered by genetically perturbing Notch signalling at multiple steps in the pathway.

Pdx1-N31C transgenic (**k–n**) pancreatic buds using antibodies specific for E-cadherin (**d, k**), carboxypeptidaseA (**e, l**), amylase (**f, m**) and ISL1 (**g, n**) show that, in the transgenic embryos, the pancreatic bud is decreased in size and poorly branched, and that exocrine, but not endocrine, cell differentiation is impaired. Scale bars in **a–c, h–j** and **d–g, k–n**, 0.1 mm.

Reduced Notch signalling leads to increased expression of the pro-endocrine gene *ngn3*, which promotes the endocrine fate. Conversely, at normal levels of Notch signalling, cells express *HES-1* and *p48* and adopt the exocrine fate. □

Methods

Animals. The generation of mice deficient in *Dll1* (ref. 10) and *RBP-Jκ* (ref. 11) has been described elsewhere and mutant mice were obtained from our local breeding colony. Mice and embryos were genotyped as described^{10,11}.

Preparation of construct for transgenic mice. A 4.5-kilobase (kb) *NotI*–*NaeI* fragment located immediately upstream of the *Ipfl/Pdx1* gene¹⁸ was sub-cloned into a vector carrying a polyA site and a 1.6-kb *BglI*–*BamHI* fragment of full-length *ngn3* complementary DNA¹² or a 2.3-kb cDNA fragment of the *Notch3* intracellular domain (*N31C*)¹³. We generated transgenic mice by pronuclear injection of the purified expression cassettes (*NotI*–*BamHI*) (2.0 ng ml^{−1}) into F2 B6/CBA hybrid oocytes as described²⁹. The genotypes were determined by PCR analyses of genomic DNA extracted from the yolk sac. The primers used were: 5′-TAGCGAGGGGGAAGAGGAGAT-3′ (*Ipfl/Pdx1* primer for 5′) and 5′-TAGGTATGAGAGTGGGGCTAGG-3′ (*ngn3* primer for 3′) for the *ngn3* transgenic mice, 5′-CAACCCAGGAGGCCCGAGTTTC-3′ (*N31C* primer for 5′) and 5′-TCGACCTGCAGGCATGCAAGC-3′ (vector primer for 3′) for the *N31C* transgenic mice.

In situ hybridization and immunohistochemistry. *In situ* hybridization, immunohistochemical localization of antigens and double-label immunohistochemistry were carried out as described². We used DIG-labelled RNA probes of *ngn3* (ref. 12), *neuroD*²¹ (provided by J. Lee), *p48* (ref. 27) (provided by O. Hagenbüchle), *Notch1* (ref. 23), *Notch2* (ref. 23), *Notch3* (ref. 23), *Dll1* (ref. 24), *Serrate1* (ref. 23) and *HES-1* (ref. 25). The primary antibodies used were rabbit anti-IPF1/PDX1 (ref. 30), rabbit anti-ISL1 (ref. 17), guinea pig anti-glucagon (Linco), rabbit anti-PYY (specific) (Euro Diagnostics), rabbit anti-amylase (Sigma), rabbit anti-carboxypeptidase A (ANAWA) and rat anti E-cadherin (Zymed). The secondary antibodies used were fluorescein anti-guinea pig (Jackson), fluorescein anti-rat (Jackson) and Cy3 anti-rabbit (Jackson).

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- Pictet, R. L., Rall, L. B., Phelps, P. & Rutter, W. J. The neural crest and the origin of the insulin-producing and other gastrointestinal hormone producing cells. *Science* **191**, 191–192 (1976).
- Fontaine, J. & Le Douarin, N. M. Analysis of endoderm formation in the avian blastoderm by the use of quail-chick chimaeras. The problem of the neuroectodermal origin of the cells of the APUD series. *J. Embryol. Exp. Morphol.* **41**, 209–222 (1977).
- Le Douarin, N. M. On the origin of pancreatic endocrine cells. *Cell* **53**, 169–171 (1988).
- Slack, J. M. W. Developmental biology of the pancreas. *Development* **121**, 1569–1580 (1995).
- Edlund, H. Transcribing pancreas. *Diabetes* **47**, 1817–1823 (1998).
- Lewis, J. Neurogenic genes and vertebrate neurogenesis. *Curr. Op. Neurobiol.* **6**, 3–10 (1996).
- Beatus, P. & Lendahl, U. Notch and neurogenesis. *J. Neurosci. Res.* **54**, 125–136 (1998).
- Pictet, R. & Rutter, W. J. In *Handbook of Physiology* (eds Steiner, D. F. & Frenkel, N.) 25–66 (Williams and Wilkins, Washington, DC, 1972).
- Ahlgren, U., Pfaff, S., Jessel, T. M., Edlund, T. & Edlund, H. Independent requirement for ISL1 in the formation of the pancreatic mesenchyme and islet cells. *Nature* **385**, 257–260 (1997).

10. Hrabe de Angelis, M., McIntyre, J. & Gossler, A. Maintenance of somite borders in mice requires the Delta homologue Dll1. *Nature* **386**, 717–721 (1997).
11. Oka, C. *et al.* Disruption of the mouse RBPJk gene results in early embryonic death. *Development* **121**, 3291–3301 (1995).
12. Sommer, L., Ma, Q. & Anderson, D. J. Neurogenins, a novel family of atonal-related bHLH transcription factors, are putative mammalian neuronal determination genes that reveal progenitor cell heterogeneity in the developing CNS and PNS. *Mol. Cell. Neurosci.* **8**, 221–241 (1996).
13. Lardelli, M., Williams, R., Mitsiadis, T. & Lendahl, U. Expression of the Notch 3 intracellular domain in mouse central nervous system progenitor cells is lethal and leads to disturbed neural tube development. *Mech. Dev.* **59**, 177–190 (1996).
14. Ma, Q., Kintner, C. & Anderson, D. Identification of neurogenin, a vertebrate neuronal determination gene. *Cell* **87**, 43–52 (1996).
15. Ma, Q., Chen, Z., del Barco Barrantes, L., de la Pompa, J. L. & Anderson, D. J. neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. *Neuron* **20**, 469–482 (1998).
16. Fode, C. *et al.* The bHLH protein NEUROGENIN 2 is a determination factor for epibranchial placode-derived sensory neurons. *Neuron* **20**, 483–494 (1998).
17. Thor, S., Ericson, J., Brannstrom, T. & Edlund, T. The homeodomain LIM protein Isl-1 is expressed in subsets of neurons and endocrine cells in the adult rat. *Neuron* **7**, 881–889 (1991).
18. Apelqvist, Å., Ahlgren, U. & Edlund, H. Sonic hedgehog directs specialised mesoderm differentiation in the intestine and pancreas. *Curr. Biol.* **7**, 801–804 (1997).
19. Upchurch, B. H., Aponte, R. G. & Liter, A. B. Peptide YY expression is an early event in colonic endocrine cell differentiation: evidence from normal and transgenic mice. *Development* **120**, 245–252 (1994).
20. Naya, F. J., Stellrecht, C. M. & Tsai, M. J. Tissue-specific regulation of the insulin gene by a novel basic helix–loop–helix transcription factor. *Genes Dev.* **9**, 1009–1019 (1995).
21. Lee, J. E. *et al.* Conversion of *Xenopus* into neurons by NeuroD, a basic helix–loop–helix protein. *Science* **268**, 836–844 (1995).
22. Naya, F. J. *et al.* Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. *Genes Dev.* **11**, 2323–2334 (1997).
23. Mitsiadis, T. A., Henrique, D., Thesleff, I. & Lendahl, U. Mouse Serrate-1 expression in the developing tooth is regulated by epithelial–mesenchymal interactions and fibroblast growth factor-4. *Development* **124**, 1473–1483 (1997).
24. Mitsiadis, T. A., Hirsinger, E., Lendahl, U. & Goridis, C. Delta–Notch signalling in odontogenesis: correlation with cytodifferentiation and evidence for feedback regulation. *Dev. Biol.* **204**, 420–431 (1998).
25. Ishibashi, M. *et al.* Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix–loop–helix factors, premature neurogenesis, and severe neural tube defects. *Genes Dev.* **15**, 3136–3148 (1995).
26. Beatus, P., Lundkvist, J., Öberg, C. & Lendahl, U. The Notch 3 intracellular domain represses Notch-mediated activation through Hairy/Enhancer of split (HES) promoters. *Development* **126**, 3925–3935 (1999).
27. Krapp, A. *et al.* The p48 DNA-binding subunit of transcription factor PTF1 is a new exocrine pancreas-specific basic helix–loop–helix protein. *EMBO J.* **15**, 4317–4329 (1996).
28. Krapp, A. *et al.* The bHLH protein PTF1-p48 is essential for the formation of the exocrine and the correct spatial organization of the endocrine pancreas. *Genes Dev.* **12**, 3752–3763 (1998).
29. Hogan, B., Constantini, F. & Lacey, E. in *Manipulating the Mouse Embryo: A Laboratory Manual* (Cold Spring Harbour Lab. Press, New York, 1994).
30. Ohlsson, H., Karlsson, K. & Edlund, T. IPF1, a homeodomain-containing transactivator of the insulin gene. *EMBO J.* **12**, 4251–4259 (1993).

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AC. elegans Ror receptor tyrosine kinase regulates cell motility and asymmetric cell division

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Ror kinases are a family of orphan receptors with tyrosine kinase activity that are related to muscle specific kinase (MuSK), a receptor tyrosine kinase that assembles acetylcholine receptors at the neuromuscular junction^{1,2}. Although the functions of Ror kinases are unknown, similarities between Ror and MuSK kinases have led to speculation that Ror kinases regulate synaptic devel-

opment. Here we show that the *Caenorhabditis elegans* gene *cam-1* encodes a member of the Ror kinase family that guides migrating cells and orients the polarity of asymmetric cell divisions and axon outgrowth. We find that tyrosine kinase activity is required for some of the functions of CAM-1, but not for its role in cell migration. CAM-1 is expressed in cells that require its function, and acts cell autonomously in migrating neurons. Overexpression and loss of *cam-1* function result in reciprocal cell-migration phenotypes, indicating that levels of CAM-1 influence the final positions of migrating cells. Our results raise the possibility that Ror kinases regulate cell motility and asymmetric cell division in organisms as diverse as nematodes and mammals.

Mutations in *cam-1* disrupt the guidance of several cells that migrate along the anteroposterior axis in *C. elegans*³. We have found that *cam-1* mutants also display defects in asymmetric cell division and axon outgrowth. Normally, six V cells on each side of first-stage larvae always divide asymmetrically, each producing an anterior daughter that joins a growing epithelial syncytium and a posterior blast cell^{4,5} (Fig. 1a, b). In *cam-1(gm122)* mutant animals, 12% of V1 cell divisions are reversed, with the anterior daughter adopting the blast-cell fate and the posterior daughter joining the syncytium (Fig. 1c, d, Table 1).

Like V cells, the six Pn.aap neuroblasts, P3.aap through P8.aap, divide asymmetrically in first-larval-stage males to generate two different daughter neurons⁶. The posterior daughters, known as CP

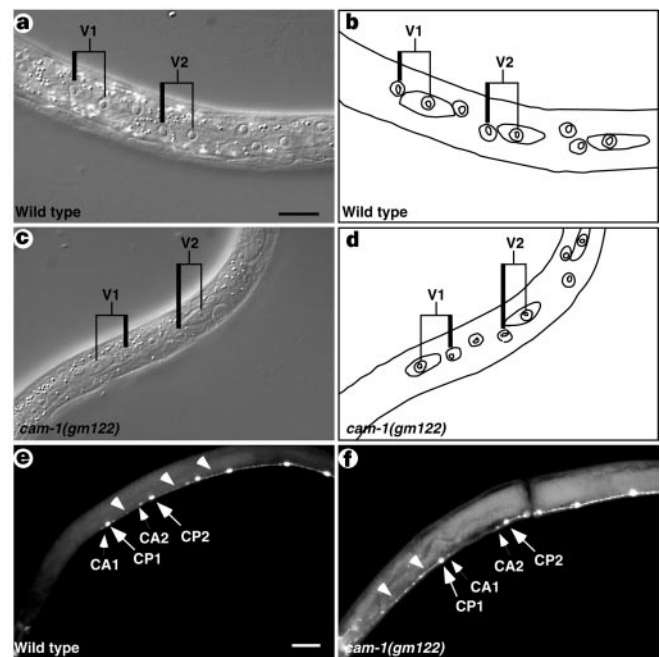


Figure 1 *cam-1* defects. Anterior is left and dorsal is up. V-cell daughters were visualized by Nomarski optics (a, c). Anterior daughters of the V cells join the epithelial syncytium so that only the outline of the nucleus can be seen. The outline of the posterior daughter cell distinguishes it from its sister. a, Wild-type L1 larva showing V1 and V2 anterior (thick line) and posterior (thin line) progeny. b, Tracing of animal in a. c, *cam-1(gm122)* L1 larva showing reversed V1 cell division. d, Tracing of animal in c. CA and CP neurons were visualized by staining with anti-serotonin antibodies (e, f). CA and CP neurons from P3.aap and P4.aap are marked. e, *him-5(e1490)* adult male showing wild-type CA and CP staining pattern. The intensely stained neurons (large arrows) are CPs. Each CP axon extends posteriorly (arrowheads). Faintly stained neurons (small arrows) just anterior to each CP may be CA7. f, *cam-1(gm122);him-5(e1490)* male showing CP1 extending its axon anteriorly (arrowheads). The faintly staining presumptive CA neuron (arrowhead) is just posterior to CP1. Scale bars in a and e represent 10 μm and 50 μm, respectively.

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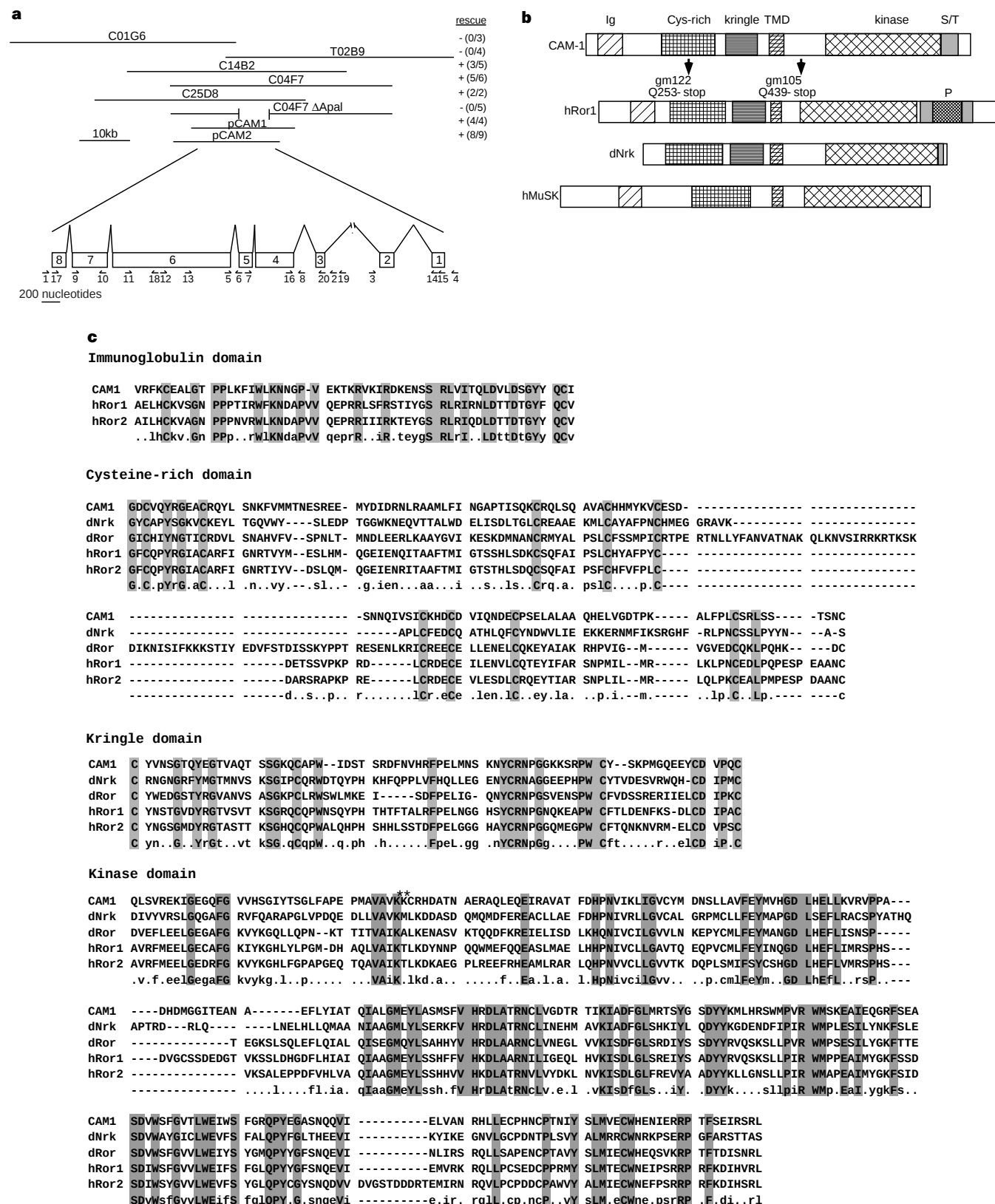


Figure 2 *cam-1* gene. **a**, Transformation rescue of *cam-1* mutants. CAN migration was scored in transgenic *cam-1(gm122)* animals bearing indicated DNAs. Column on the right indicates whether the DNA rescues (+) or doesn't rescue (-). The number in parentheses is the ratio of lines that rescued. Below is the *cam-1* gene structure. Transcription is from right to left. Exons are indicated by boxes and introns by lines. The second intron is not drawn to scale. Arrows at the bottom represent primers used in PCR experiments. 1 refers to *cam1*, 2 to *cam2*, and so on. **b**, Comparison of CAM-1, a human Ror (hRor1), a *Drosophila* Ror (dNrK), and

human MuSK (hMuSK). Arrows indicate sites of *cam-1* mutations. **c**, Alignments of domains. CAM-1, *Drosophila* Rors (dNrK and dRor) and human Rors (hRor1 and hRor2) were aligned using the MAP alignment algorithm²⁵. Asterisks indicate two lysines that were mutated in pCAM-1-GFP*. At the bottom is a consensus sequence. Uppercase indicates amino acids found in all proteins shown. Lowercase indicates amino acids found in at least two (immunoglobulin domains) or three (all other domains) of the proteins shown.

neurons, express high levels of the neurotransmitter serotonin and extend an axon posteriorly to the tail. The anterior daughters, known as CA neurons, appear to accumulate low levels of serotonin⁷. Staining of wild-type males with an anti-serotonin antiserum revealed the six CP neurons and their posteriorly directed axons, as well as weakly staining cells, presumably CAs, just anterior to the four most anterior CP neurons (Fig. 1e). Instead of extending its axon posteriorly, the most anterior CP neuron in 26–55% of *cam-1* mutants extended its axon anteriorly to the head (Fig. 1e, f, Table 1). We also occasionally detected a weakly staining serotonergic neuron just posterior to the most anterior CP neuron, indicating that polarity of the P3.aap precursor division may have been reversed (Fig. 1f, Table 1). Anteriorly directed CP axons and putative CA/CP reversals often occurred independently of one another. For example, a P3.aap could divide normally to produce a posterior CP daughter with an anteriorly directed axon.

We identified DNAs that rescued the migration and V-cell-polarity defects of two *cam-1* mutants, and found that *cam-1* encodes a receptor tyrosine kinase that is most similar to members of the Ror kinase family (Fig. 2). Ror genes have been identified in humans and *Drosophila*. In both organisms, their functions are unknown^{8–10}. The kinase domain of CAM-1 is about 45% identical and 72% similar to those of vertebrate and *Drosophila* Rors. In addition to their highly related kinase domains, the human Rors and CAM-1 contain similar extracellular domains: an immunoglobulin domain, a cysteine-rich domain and a kringle domain (Fig. 2b, c). CAM-1 also contains an intracellular serine-rich region found in human Rors (Fig. 2b).

Sequencing *cam-1* genes from the mutants revealed that both mutant alleles are nonsense mutations (Fig. 2b). *gm122* lies within the extracellular cysteine-rich domain, consistent with genetic results indicating that *gm122* may eliminate *cam-1* function¹¹. *gm105* is a recessive mutation that is predicted to truncate the protein by 24 amino acids amino-terminal to the kinase domain. Like *gm122*, *gm105* disrupts locomotion, dauer development and CP axon outgrowth but, unlike *gm122*, it has no effect on V1 division and only subtle effects on cell migrations^{3,11} (see above). *gm105* phenotypes indicate that kinase activity may not be required for all CAM-1 functions. To test this possibility, we changed a conserved lysine in the ATP-binding domain to arginine, an alteration predicted to eliminate kinase activity¹², and introduced the mutant transgene (pCAM-1-GFP*) into *gm122* animals. Expression of the mutant and wild-type transgenes is identical

(see below). pCAM-1-GFP* rescued the cell-migration defects of *gm122* hermaphrodites (not shown), consistent with the hypothesis that kinase activity is not necessary for CAM-1 function in cell motility. Kinase-independent functions have been described for both cytoplasmic tyrosine kinases¹³ and receptor tyrosine kinases¹⁴. The transgene failed to rescue the V1 defects in *gm122* mutants, which was unexpected, as *gm105* mutants did not show these defects (Table 1). The transgene result indicates that kinase activity may be necessary for V1 division. If this hypothesis were correct, *gm105* animals would produce some full-length CAM-1, either by alternate splicing or readthrough. *gm122* transgenic animals also showed defects in V2 and V4 that were not seen in *cam-1* mutants, indicating that pCAM-1-GFP* may possess an altered function (Table 1). Because pCAM-1-GFP* does not cause V-cell defects in the wild type (data not shown), wild-type *cam-1* may antagonize the abnormal activity of pCAM-1-GFP*.

To determine the expression pattern of CAM-1, we generated animals bearing a functional *cam-1-gfp* transgene that rescues the defects of *cam-1* mutants. CAM-1-GFP expression appears at the 200-cell stage in most cells of the embryo (Fig. 3a). Migrating ALM, BDU, CAN, HSN and ccM cells, which migrate embryonically and require *cam-1* function for their migration, are likely to express CAM-1-GFP, as most cells of the embryo express the transgene while these cells migrate. During the first larval stage, V cells often express CAM-1-GFP at the time that they divide (Fig. 3c), and the Q-neuroblast descendants, which also require *cam-1* function for their migration, express CAM-1-GFP (not shown). During larval development, CAM-1-GFP is highly expressed in the nervous system, as well as in intestinal, hypodermal and body-wall muscle cells and in parts of the pharynx (Fig. 3b–d). In non-neuronal cells, much of the protein appears to associate with the plasma membrane. In neurons, CAM-1-GFP is detected predominantly in axons and dendrites (Fig. 3c, d).

Because CAM-1 is expressed in so many cell types, we used mosaic analysis to investigate whether *cam-1* acts in cells that require its function^{15–17} (Fig. 4). In the wild type, the CANs migrate posteriorly from the head to the centre of the embryo¹⁸. In *cam-1* mutants, the CANs usually fail to migrate and are displaced anteriorly³. To test whether *cam-1* functions cell autonomously in the CANs, we generated animals that were mutant for *cam-1* and

Table 1 *cam-1* mutant asymmetric cell division and axon outgrowth defects

Strain†	Percent V-cell polarity reversals‡					
	V1	V2	V3	V4	V5	V6
Wild type	0 (46)	0 (46)	0 (46)	0 (46)	0 (46)	0 (46)
<i>cam-1(gm105)</i>	0 (55)	0 (55)	0 (55)	0 (55)	0 (55)	0 (55)
<i>cam-1(gm122)</i>	12 (60)	0 (60)	0 (60)	0 (60)	0 (60)	0 (60)
<i>cam-1(gm122)</i> + pCAM-1-GFP	0 (27)	0 (27)	0 (27)	0 (27)	0 (27)	0 (27)
<i>cam-1(gm122)</i> + pCAM-1-GFP*	10 (48)	4 (48)	0 (48)	2 (48)§	0 (48)	0 (48)
	Percent CA/CP polarity reversals					Anterior axon
	Ca/CP1	CA/CP2	CA/CP3	CA/CP4		
Wild type	0 (15)	0 (15)	0 (16)	0 (19)	0 (28)	
<i>cam-1(gm105)</i>	32.1 (28)	0 (34)	0 (27)	0 (18)	25.9 (58)	
<i>cam-1(gm122)</i>	35.0 (20)	4.8 (21)	0 (19)	0 (15)	55.2 (29)	

The table shows cell-polarity defects in *cam-1* mutants. The number of cells or axons examined is shown in parentheses.

† Both pCAM-1-GFP and pCAM-1-GFP* DNAs are present as extrachromosomal arrays. Neither pCAM-1-GFP nor pCAM-1-GFP* caused any V-cell defects in a wild-type background (not shown).

‡ Percentage of V-cell divisions with reversed polarity (V1–V6).

§ The abnormal V cell divided symmetrically to generate two daughter cells that joined the hypodermal syncytium.

|| Percentage of C1–C4 cell divisions with reversed polarity (CA/P1–CA/P4) and animals in which an axon extended anteriorly towards the head (anterior axon).

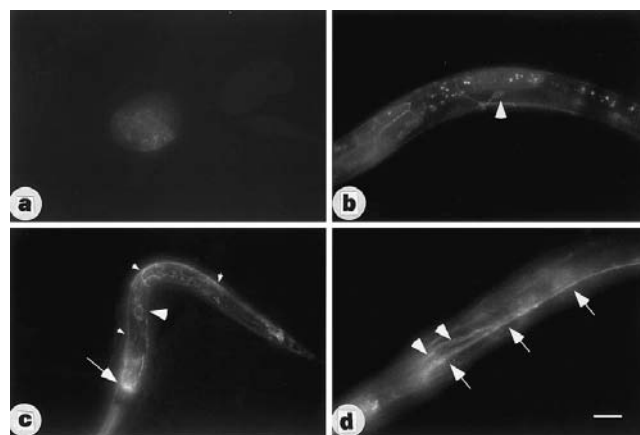


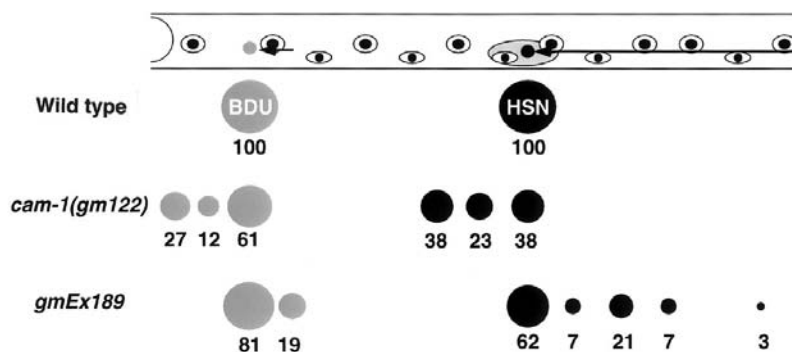
Figure 3 *cam-1-gfp* transgene expression. Anterior is to the left and dorsal is up. **a**, CAM-1 is expressed in most cells of a 250-min embryo. **b**, CAM-1 is sometimes expressed in neuron soma (arrowhead points to the HSN). **c**, Lateral hypodermal cells including V1 (large arrowhead) express CAM-1 in the first larval stage. Protein is predominantly membrane associated. Arrow indicates CAM-1 in the nerve ring, the major *C. elegans* neuropil. Small arrowheads indicate the dorsal nerve cord. **d**, CAM-1 is expressed in muscle cells and is predominantly membrane associated. Arrowheads indicate the dorsal edge of a ventral muscle cell. Arrows indicate the position of the ventral nerve cord. Scale bar, 10 μm.

To overexpress CAM-1, we generated animals that contained the *cam-1* gene at high copy number. The migrations of the CAN and ALM neurons and the polarity of the V-cell divisions, all processes that require *cam-1* function, were normal in these animals. In contrast, the anterior migrations of the BDU and HSN neurons often terminated prematurely. Loss of *cam-1* function causes the BDU and HSN neurons to migrate beyond their normal destinations, a phenotype reciprocal to that caused by CAM-1 overexpression (Fig. 5). Consistent with the hypothesis that kinase activity is not necessary for CAM-1's role in cell motility, BDU and HSN migrations also terminated prematurely in animals that overexpress the kinase mutant pCAM-1-GFP* (data not shown). These results suggest that CAM-1 activity, and perhaps a graded distribution of the CAM-1 ligand, may regulate the final position of migrating cells.

The pedigree chart illustrates the inheritance of the CAN gene across several generations. The starting point is a 'zygote' which leads to a parent generation with genotypes AB and P1. The AB parent is a mosaic animal with both CANs misplaced (indicated by three black circles). The P1 parent is a mosaic animal with both CANs positioned normally (indicated by a white square). The chart shows the segregation of these alleles through various crosses, with labels 'a', 'p', 'r', 'l', 'EMS', and 'P2' indicating specific genetic events or genotypes. The final generation shows the segregation of the CAN gene into 'CANL' and 'CANR' alleles. A legend box provides the key for the symbols used: black circle for 'Mosaic animal with both CANs misplaced', black circle with 'L' for 'Mosaic animal with left CAN misplaced', black circle with 'R' for 'Mosaic animal with right CAN misplaced', and white square for 'Mosaic animal with CANs positioned normally'.

- Mosaic animal with both CANs misplaced
- ⬤ Mosaic animal with left CAN misplaced
- ⊗ Mosaic animal with right CAN misplaced
- Mosaic animal with CANs positioned normally

Methods. Four of the six losses depicted as ABpl losses occurred later in this lineage; two were at ABplpa and two were at ABplap. Because EMS divides to produce E and MS, and because we could not score the Ncl marker in cells derived from E, losses in EMS could have occurred in MS. We scored transmission of the array to the next generation to determine whether mosaic animals had lost the array in the P2 lineage. Because six of the EMS mosaic losses resulted in lethality or sterility, these losses could have occurred in P1.



migration route. The area of each circle in the chart is proportional to the percentage of BDUs (grey circles) or HSNs (black circles) in that position along the anteroposterior axis of L1 larva. The percentage represented by each is shown below the circle. Strains scored were N2 (wild type), the *cam-1(gm122)* mutant, and a wild-type strain that carries the *cam-1* overexpressing transgene *gmEx189*.

these processes is polarity. Migrating cells and growth cones are overtly polarized, with a leading edge that contains a zone of actively polymerizing filamentous actin^{19,20}. Blast cells can be polarized, containing asymmetrically localized molecules that distribute developmental potential to daughter cells²¹. How these cells become polarized, however, is poorly understood. Our results suggest that cell signalling through receptor tyrosine kinases regulates the polarity involved in cell motility and asymmetric cell division.

Rors had been identified previously as orphan receptor tyrosine kinases that are expressed in mammalian and *Drosophila* nervous systems. The conservation between CAM-1 and other family members suggests that Rors may regulate cell motility and the distribution of cell fate during asymmetric cell division in organisms other than *C. elegans*. □

Methods

Cell polarity defects. V-cell daughters were examined using Nomarski optics⁴. We also followed the division of V cells in six animals. Two V1 divisions were reversed. We did not detect aberrant cell migration or extra cell divisions during the aberrant divisions. CA and CP neurons were examined by indirect immunofluorescence after staining males with an anti-serotonin antiserum²².

Cell-migration defects. We assessed the positions of BDU and HSN by Nomarski optics. BDU migrates from a position just posterior of V1 to a position just anterior of V1 (J. Sulston, personal communication), and HSN migrates from the tail to the side of the gonad primordium¹⁸ (Fig. 5). We assumed that when we could not detect BDU, it had migrated into a region where we could not distinguish it from other neurons. In animals bearing *gmEx189* or *gmEx190*, we scored BDUs and HSNs in larvae that expressed TTX-3-GFP. We scored approximately 30 BDUs and 30 HSNs for each strain.

Cloning. *cam-1* mutations were mapped on chromosome II between *let-23* and *mel-11*. Cosmids containing chromosomal DNA from this interval were injected into *gm122* animals²³, and transgenic progeny were scored for rescue of *Cam-1* phenotypes (Fig. 2a). Deletion of a 6992-base pair (bp) fragment from the rescuing cosmid C04F7 (C04F7ΔApal) abolished the ability of that cosmid to rescue. Subclones pCAM-1 (20.9-kilobase (kb) *NheI* fragment of C25D8 cloned into pBluescript KS+) and pCAM-2 (23.3-kb *PstI* fragment of C25D8 cloned into pBluescript KS+), which contained only one predicted gene from the region, D2013.4, the receptor tyrosine kinase gene, rescued *cam-1* mutants.

Sequencing *cam-1* mutants. We sequenced D2013.4 from both *cam-1* mutants. We used polymerase chain reaction (PCR) with primers cam3 and cam4, cam2 and cam13, and cam1 and cam6 to produce products that were cloned into pBluescript KS+ (Fig. 2a). Three clones from independent PCRs were sequenced using internal primers.

Gene structure. To determine the *cam-1* gene structure, we performed reverse transcription, followed by PCR using primers cam12 and cam14, cam12 and cam15, cam12 and cam20, cam11 and cam14, cam11 and cam15, and cam11 and cam20 (Fig. 2a). Each pairwise combination of primers produced a single product that was sequenced. The 3' gene structure was determined by sequencing complementary DNA clones yk17c2 and yk16c11.

Mosaic analysis. We performed mosaic analysis as described^{15–17}. Animals of the genotype *cam-1(gm122); ncl-1(e1865); klys5[ceh-23-unc-76-gfp; lin-15]; gmEx188[pCAM-1; ncl-1(+); pRF4rol-6(dm)]* were generated by microinjecting 33 μg ml⁻¹ pCAM-1, 100 μg ml⁻¹ pRF4 and 33 μg ml⁻¹ C33C3 (containing the wild-type *ncl-1* gene) into *cam-1(gm122); ncl-1(e1865); klys5[ceh-23-unc-76-gfp; lin-15]* hermaphrodites²³. We used two strategies to identify mosaic animals. First, we screened for Rol animals with misplaced CANs, and then we determined by Ncl where in the lineage the array was lost. In these animals, 33 CANs were misplaced and 32 of these were Ncl⁻, indicating that the misplaced CANs had lost the array. One of the 33 misplaced CANs was wild-type for *ncl-1*, consistent with the observation that rescue of the CAN cell-migration defect by *gmEx188* is incomplete (not shown). Second, we screened directly for mosaic animals by Nomarski optics, and then scored the positions of the CANs in these animals. All the CANs in this group were positioned normally. Two CANs were Ncl⁻, indicating that they had lost the array. The two CANs that were positioned normally despite having lost the array reflect the incomplete penetrance of the CAN-migration defect in *gm122* animals⁵. Several mosaic animals had lost the

array multiple times. The multiple losses were ABa and ABplpapa; ABa and EMS; ABa and ABalppppa; ABa and ABpraa; ABalapa and ABalppppa; ABalapa and ABplp and EMS (normal CANs); ABalapp and EMS (normal CANs); ABalapp and ABprap; ABalapp and ABalpppp; ABpr and EMS; EMS and ABprpp.

GFP transgenes. To generate pCAM-1-GFP, a 3220-bp fragment was amplified using the PCR primers cam17 and cam19 using TaqPlus (Stratagene). The PCR product was cloned into pPD.75 (A. Fire, personal communication) to generate pWF5. A 8169-bp *PstI*-*BglII* fragment from pCAM-1 was ligated into pWF5 to generate pCAM-1-GFP. To generate pCAM-1-GFP*, a PCR product was generated using a primer containing the mutant codons and was cloned into pCAM-1-GFP. These genes were sequenced to confirm the absence of PCR errors.

Transgenic strains. To generate reporter transgenes *gmEx191* and *gmEx199*, pCAM-1-GFP or pCAM-1-GFP*, respectively, were injected into the wild type at 50 μg ml⁻¹ along with the marker pRF4 [*rol-6(su1006)*]²³ at 100 μg ml⁻¹. To overexpress CAM-1, *gmEx189* was generated by injecting pCAM-1 at high concentration (150 μg ml⁻¹) along with *ttx-3-gfp*²⁴ and pRF4. pCAM-1ΔNcol-fill in was generated by cleaving pCAM-1 with *NcoI*, treating the ends with Klenow enzyme and re-ligating to introduce a 102-nucleotide deletion from sequences encoding the kringle domain and a +1 frameshift.

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- Valenzuela, D. M. *et al.* Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. *Neuron* **15**, 573–584 (1995).
- DeChiara, T. M. *et al.* The receptor tyrosine kinase MuSK is required for neuromuscular junction formation *in vivo*. *Cell* **85**, 501–512 (1996).
- Forrester, W. C. & Garriga, G. Genes necessary for *C. elegans* cell and growth cone migrations. *Development* **124**, 1831–1843 (1997).
- Sulston, J. E. & Horvitz, H. R. Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Dev. Biol.* **56**, 110–156 (1977).
- Podbilewicz, B. & White, J. G. Cell fusions in the developing epithelial of *C. elegans*. *Dev. Biol.* **161**, 408–424 (1994).
- Sulston, J. E., Albertson, D. G. & Thomson, J. N. The *Caenorhabditis elegans* male: postembryonic development of nongonadal structures. *Dev. Biol.* **78**, 542–576 (1980).
- Loer, C. M. & Kenyon, C. J. Serotonin-deficient mutants and male mating behavior in the nematode *Caenorhabditis elegans*. *J. Neurosci.* **13**, 5407–5417 (1993).
- Masiakowski, P. & Carroll, R. D. A novel family of cell surface receptors with tyrosine kinase-like domain. *J. Biol. Chem.* **267**, 26181–26190 (1992).
- Wilson, C., Gubergh, D. C. & Steller, H. Dror, a potential neurotrophic receptor gene, encodes a *Drosophila* homolog of the vertebrate Ror family of Trk-related receptor tyrosine kinases. *Proc. Natl Acad. Sci. USA* **90**, 7109–7113 (1993).
- Oishi, I. *et al.* A novel *Drosophila* receptor tyrosine kinase expressed specifically in the nervous system. Unique structural features and implication in developmental signaling. *J. Biol. Chem.* **272**, 11916–11923 (1997).
- Forrester, W. C., Perens, E., Zallen, J. A. & Garriga, G. Identification of *Caenorhabditis elegans* genes required for neuronal differentiation and migration. *Genetics* **148**, 151–165 (1998).
- Hanks, S. K., Quinn, A. M. & Hunter, T. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241**, 42–52 (1988).
- Henkemeyer, M., West, S. R., Gertler, F. B. & Hoffmann, F. M. A novel tyrosine kinase independent of *Drosophila* abl correlates with proper subcellular localization. *Cell* **63**, 949–960 (1990).
- Apel, E. D., Glass, D. J., Moscoso, L. M., Yancopoulos, G. D. & Sanes, J. R. Rapsyn is required for MuSK signaling and recruits synaptic components to a MuSK-containing scaffold. *Neuron* **18**, 623–635 (1997).
- Herman, R. K. Analysis of genetic mosaics of the nematode *Caenorhabditis elegans*. *Genetics* **108**, 165–180 (1984).
- Hedecock, E. M. & Herman, R. K. The *ncl-1* gene and genetic mosaics of *Caenorhabditis elegans*. *Genetics* **141**, 989–1006 (1995).
- Miller, L. M., Waring, D. A. & Kim, S. K. Mosaic analysis using a *ncl-1* (+) extrachromosomal array reveals that *lin-31* acts in the Pn.p cells during *Caenorhabditis elegans* vulval development. *Genetics* **143**, 1181–1191 (1996).
- Sulston, J. E., Schierenberg, E., White, J. G. & Thomson, J. N. The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **100**, 64–119 (1983).
- Lauffenburger, D. A. & Horvitz, A. F. Cell migration: a physically integrated molecular process. *Cell* **84**, 359–369 (1996).
- Mitchison, T. J. & Cramer, L. P. Actin-based cell motility and cell locomotion. *Cell* **84**, 371–379 (1996).
- Hawkins, N. & Garriga, G. Asymmetric cell division: from A to Z. *Genes Dev.* **12**, 3625–3638 (1998).
- McIntire, S. L., Garriga, G., White, J., Jacobson, D. & Horvitz, H. R. Genes necessary for directed axonal elongation or fasciculation in *C. elegans*. *Neuron* **8**, 307–322 (1992).
- Mello, C. C., Kramer, J. M., Stinchcomb, D. & Ambros, V. Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* **10**, 3959–3970 (1991).
- Hoebert, O. *et al.* Regulation of interneuron function in the *C. elegans* thermoregulatory pathway by the *ttx-3* LIM homeobox gene. *Neuron* **19**, 345–357 (1997).
- Huang, X. On global sequence alignment. *Comput. Appl. Biosci.* **10**, 227–235 (1994).

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Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis

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The protein Bid is a participant in the pathway that leads to cell death (apoptosis), mediating the release of cytochrome *c* from mitochondria in response to signals from 'death' receptors known as TNFR1/Fas on the cell surface^{1–7}. It is a member of the pro-apoptotic Bcl-2 family⁸ and is activated as a result of its cleavage by caspase 8, one of a family of proteolytic cell-death proteins. To investigate the role of Bid *in vivo*, we have generated mice deficient for Bid. We find that when these mice are injected with an antibody directed against Fas, they nearly all survive, whereas wild-type mice die from hepatocellular apoptosis and haemorrhagic necrosis. About half of the *Bid*-deficient animals had no apparent liver injury and showed no evidence of activation of the

effector caspases 3 and 7, although the initiator caspase 8 had been activated. Other Bid-deficient mice survived with only moderate damage: all three caspases (8 and 37) were activated but their cell nuclei were intact and no mitochondrial cytochrome *c* was released. We also investigated the effects of Bid deficiency in cultured cells treated with anti-Fas antibody (hepatocytes and thymocytes) or with TNF α (fibroblasts). In these *Bid*^{–/–} cells, mitochondrial dysfunction was delayed, cytochrome *c* was not released, effector caspase activity was reduced and the cleavage of apoptosis substrates was altered. This loss-of-function model indicates that Bid is a critical substrate *in vivo* for signalling by death-receptor agonists, which mediates a mitochondrial amplification loop that is essential for the apoptosis of selected cells.

To assess the role of Bid *in vivo*, we constructed a targeting vector that deleted a 4.2-kilobase (kb) *PstI/NheI* *Bid* murine genomic fragment containing exons 1 and 2 and part of exon 3, which encodes the BH3 domain (Fig. 1a)⁹. Two of the independent, homologous recombinant clones of RW-4 embryonic stem (ES) cells transmitted the disrupted allele to the germline (Fig. 1b, c). The absence of Bid expression in *Bid*^{–/–} tissues was demonstrated by immunoblotting using a polyclonal anti-Bid antibody (Fig. 1d). *Bid*^{–/–} mice were born at the expected mendelian frequency, had no apparent developmental abnormalities, and had normal spleens, thymuses, brains, hearts, livers, lungs, kidneys and testes with respect to weight and histology (data not shown).

The engagement of Fas/TNFR1 death receptors^{10,11} leads to the formation of a protein complex known as DISC (or death-inducing signalling complex) and to the activation of caspase 8 (refs 12, 13), which cleaves the larger (*M_r* 22K) cytosolic p22 Bid to its active form, p15 Bid, which translocates into the mitochondrion as an integral membrane protein^{1–3}. p15 Bid has been implicated as a

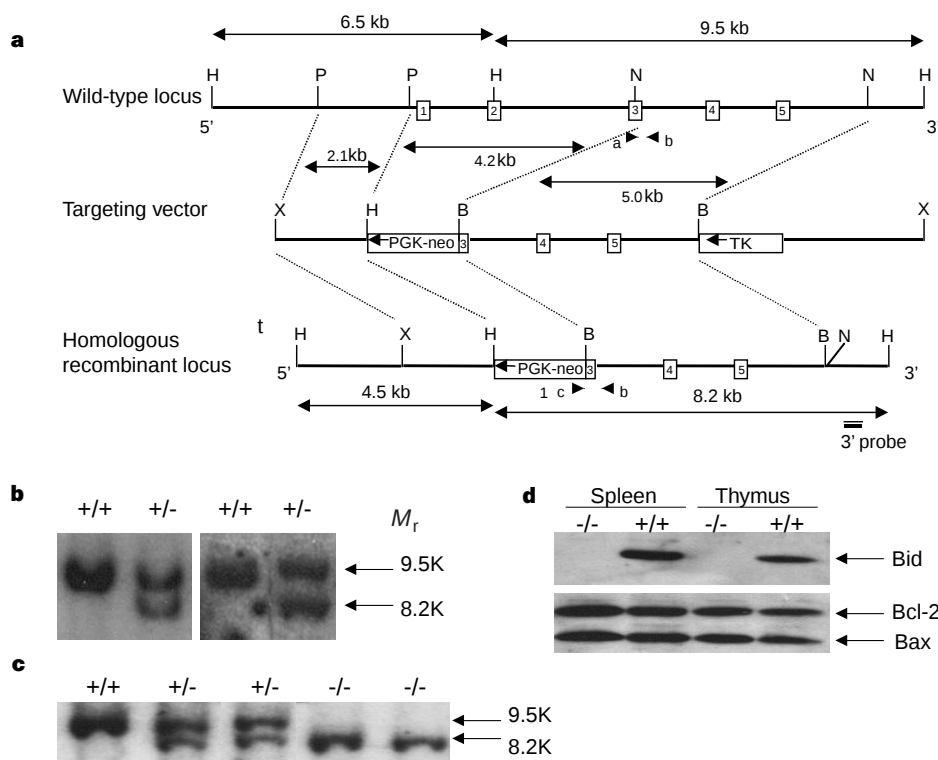


Figure 1 Generation of *Bid*^{–/–} mice by gene targeting. **a**, Diagram of the *Bid* genomic locus, the targeting vector and the homologous recombinant. Also indicated are the restriction enzyme cutting sites (H; *HindIII*; P; *PstI*; N; *NheI*; X; *XhoI*; B; *BamHI*) and the position of a 3' external probe. Arrowheads labelled a, b and c indicate the positions of primers used in screening genotypes by PCR. **b**, Southern blot demonstrating homologous recombination within the *Bid* locus

of the two ES clones that subsequently transmitted to the germ line. The 3' external probe detects a 9.5-kb *HindIII* fragment for wild-type loci and a 8.2-kb *HindIII* recombinant fragment. **c**, Genotypes of mouse progeny using the same Southern strategy. **d**, Western blot demonstrating the absence of Bid protein but the presence of Bcl-2 and Bax in *Bid*^{–/–} mice.

Table 1 Resistance of *Bid*^{-/-} mice to anti-Fas-induced mortality and hepatocyte injury

Mice	Survival at 48 h (%)	Survival time of deceased animals (h) (mean ± s.d.)	2h (mean ± s.d.)	Histology scores* 2.5–3.5 h	6–9 h
WT	12.5% (n = 8)	3.2 ± 1.3 (n = 7)	1, 2, 3, 3† (2.3 ± 1.0)	3‡, 3‡	3‡
<i>Bid</i> ^{-/-}	81.8% (n = 11)	6.6 ± 1.3 (n = 2)	0, 0, 1, 0† (0.3 ± 0.5)	0†, 1†	3‡
P††	<0.01	0.01	0.01	–	–

* Semiquantitative histological scoring of liver injury (see Methods). Data shown are scores for each mouse and also mean ± s.d. for animals killed at the 2-h timepoint.

† WT and *Bid*^{-/-} mice were killed at matched timepoints for comparison.

‡ Animals were killed when they became symptomatic.

†† Statistics were performed using χ^2 test for survival rate, *t*-test for mean survival time and mean histology score.

component required for the release from mitochondria of cytochrome *c* (refs 1, 3). To determine whether Bid is required *in vivo* for cytochrome *c* release, mitochondrial dysfunction or the death of cells, we injected mice intravenously with anti-Fas antibody (Jo2; 0.25 $\mu\text{g g}^{-1}$)¹⁴. Most wild-type (WT) mice (7/8) died within ~4 h of acute liver failure associated with massive hepatic apoptosis and haemorrhagic necrosis (Fig. 2; Table 1). Within 2 h of injection, the initiator caspase 8 and the effector caspases 3 and 7 were activated, as evidenced by immunohistochemical staining of livers for caspases 3 and 7 (Figs 3b, 4b), immunoblotting (Fig. 3g) and cleavage of selective peptide substrates (Fig. 3h)¹⁵. Cytochrome *c* immunoreactivity was lost in wild-type hepatocytes undergoing apoptosis, reflecting either release and degradation or a conformational change in cytochrome *c* (Figs 3a, 4a)^{16,17}. In contrast, most *Bid*^{-/-} mice (9/11) survived anti-Fas antibody injection (Fig. 2a), about half had no apparent liver injury by gross or microscopic examination (Fig. 2b, c; Table 1), and there was no evidence of apoptosis from nuclear morphology (Fig. 3e) or TUNEL staining (Fig. 3f compared to 3c). Analysis of this subset of *Bid*-deficient livers indicated that caspase 8 had been activated (Fig. 3g, h) but that caspases 3 and 7 had not, as shown by analysis *in situ* (Fig. 3e), immunoblotting (Fig. 3g) and cleavage of the DEVD peptide substrate (Fig. 3h); nor had cytochrome *c* been released or its immunoreactivity lost in these *Bid*-deficient hepatocytes (Fig. 3d). These unaffected mice thus require a Bid-mediated pathway for effective effector-caspase activity and for mitochondrial dysfunction to cause apoptotic damage.

Other *Bid*^{-/-} mice had moderate liver injury which they often survived; however, the two that died survived longer (6.6 h) than wild-type mice (3.2 h) (Fig. 2a; Table 1). *Bid*-deficient mice may suffer hepatocyte injury depending on the effectiveness of delivery of anti-Fas antibody or perhaps on the variable presence of a modifier locus. Using immunohistochemical analysis of affected regions of *Bid*^{-/-} livers, we found that, although caspases 3 and 7 were activated (Fig. 4f), cytochrome *c* immunoreactivity was often retained (Fig. 4e) and nuclei were not substantially condensed or fragmented (Fig. 4g) compared with wild-type livers (Fig. 4a–c).

Co-staining for activated caspases 3 and 7 and for cytochrome *c*, together with nuclear morphology, confirmed that damage in affected areas of *Bid*^{-/-} livers was mild. Wild-type hepatocytes had activated caspases 3 and 7 in the same cells that had lost cytochrome *c* immunoreactivity and their nuclei were condensed or fragmented (Fig. 4d); however, affected *Bid*^{-/-} hepatocytes activated caspases 3 and 7 in the presence of cytochrome *c* immunoreactivity and relatively normal nuclei (Fig. 4h). At this 2-h timepoint, the subcellular distribution of activated caspases 3 and 7 in *Bid*^{-/-} hepatocytes was largely restricted to the cell periphery (Fig. 4h).

We investigated the Bid-dependent component of apoptosis in cultured hepatocytes, embryonic fibroblasts (MEFs) and thymocytes. Cultured *Bid*-deficient hepatocytes survived for longer after treatment with anti-Fas antibody and cycloheximide, although they died later in the presence of cycloheximide (Chx) (Fig. 5a). *Bid*^{-/-} hepatocytes treated *in vitro* showed a delay in caspase-8 activation, corrected by 10 h, whereas the activity of caspase 3 was diminished throughout the time course of the experiment, as indicated by immunoblotting (Fig. 5b) and peptide cleavage (results not shown). Although MEFs and thymocytes do not absolutely require Bid for TNF- α /Fas-induced death, *Bid* deficiency delays apoptosis (Fig. 5c) and we were able to make a biochemical comparison. *Bid*-deficient thymocytes displayed more resistance to recombinant Fas ligand (FasL) as well as to anti-Fas antibody. *Bid*^{-/-} MEFs treated with TNF- α did not release substantial amounts of cytochrome *c* from mitochondria (Fig. 5d); caspase 8 was activated in *Bid*^{-/-} MEFs and thymocytes treated with TNF- α or anti-Fas antibody, respectively (Fig. 5e). Activation of caspases 3 and 7 was delayed and diminished in *Bid*-deficient MEFs and thymocytes, as assessed by immunoblotting (results not shown) and DEVDase activity (Fig. 5e). At later time points, *Bid*-deficient MEFs had less caspase-8-selective LET-Dase activity (Fig. 5e), which may reflect defective feed-forward cleavage of caspase-8 following onset of mitochondrial dysfunction¹⁸. There was less cleavage of some downstream substrates in *Bid*-deficient cells (Fig. 5f), including polyADP-ribose polymerase (PARP) and ICAD^{19,20}, which are nuclear components

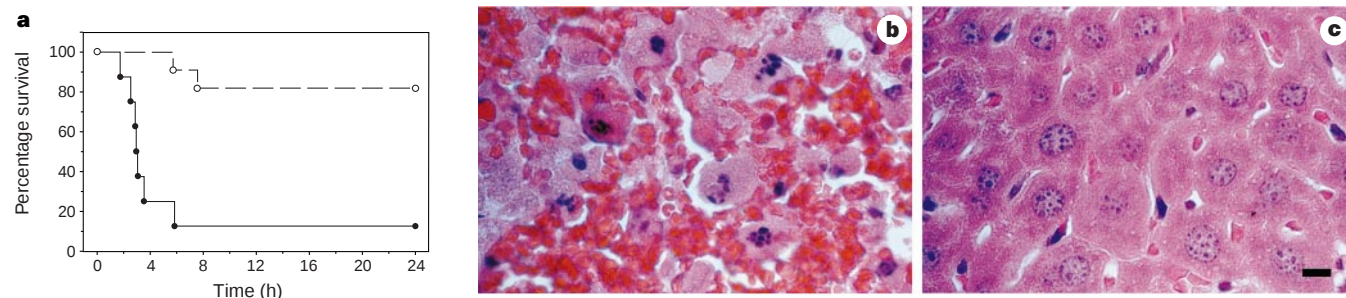


Figure 2 Resistance of *Bid*^{-/-} mice to anti-Fas-antibody-induced mortality and hepatic injury. **a**, Survival curve of wild-type (WT; filled circles, *n* = 8) and *Bid*^{-/-} mice (circles, *n* = 11) in response to a single i.v. injection of 0.25 $\mu\text{g g}^{-1}$ anti-Fas antibody (Jo-2). This represents the survival curve of the best-matched cohort

from a total of 24 WT and 40 *Bid*^{-/-} mice that were injected with anti-Fas antibody. **b, c**, Haematoxylin-eosin staining of paraffin-embedded liver sections of WT (**b**) and *Bid*^{-/-} mouse (**c**) 2 h after anti-Fas antibody injection. Note the condensed and fragmented nuclei and the haemorrhage in the WT liver. Scale bar, 25 μm .

of the apoptosis pathway—this may bear on the retention of nuclear morphology in *Bid*^{-/-} cells that have started apoptosis (Fig. 4g, h). Cleavage of the cytosolic 14-3-3 protein was slightly delayed in *Bid*^{-/-} compared with +/+ cells, but was similar in both for the cytoskeletal fodrin molecule (Fig. 5f). These differences in the pattern and time course of substrate cleavage may relate to the peripheral distribution as well as the delayed activation of caspases 3 and 7 in *Bid*^{-/-} cells (Fig. 4f, h).

Mitochondrial dysfunction occurs in the Bid-dependent pathway. In the absence of Bid to translocate to the mitochondria, the transmembrane potential ($\Delta\Psi_M$) of mitochondria is better maintained in *Bid*-deficient MEFs treated with TNF- α or in thymocytes treated with anti-Fas antibody (Fig. 5c). In contrast, other stimulators of cell death such as staurosporine, dexamethasone and γ -

radiation (results not shown), which do not signal primarily through TNFR1/Fas, gave comparable alterations of $\Delta\Psi_M$ and survival of *Bid*^{+/-} and -/- cells (Fig. 5c).

Our *Bid*-deficient mice demonstrate that Bid is an indispensable substrate for select cells and death agonists, as shown by the hepatocyte apoptosis induced by Fas *in vivo*. The subset of null mice that had no liver injury was the most striking. Hepatocytes may resemble type-II cell lines in which Fas-induced death is dependent on mitochondria¹⁸. Consistent with this, a *Bcl-2* transgene protected liver from Fas-induced apoptosis²¹, although this effect may be dose-dependent²². Cultured hepatocytes show more delay in activation of caspase-8 than do *in situ* hepatocytes, although both require a Bid-dependent mitochondrial loop to activate sufficient effector caspases.

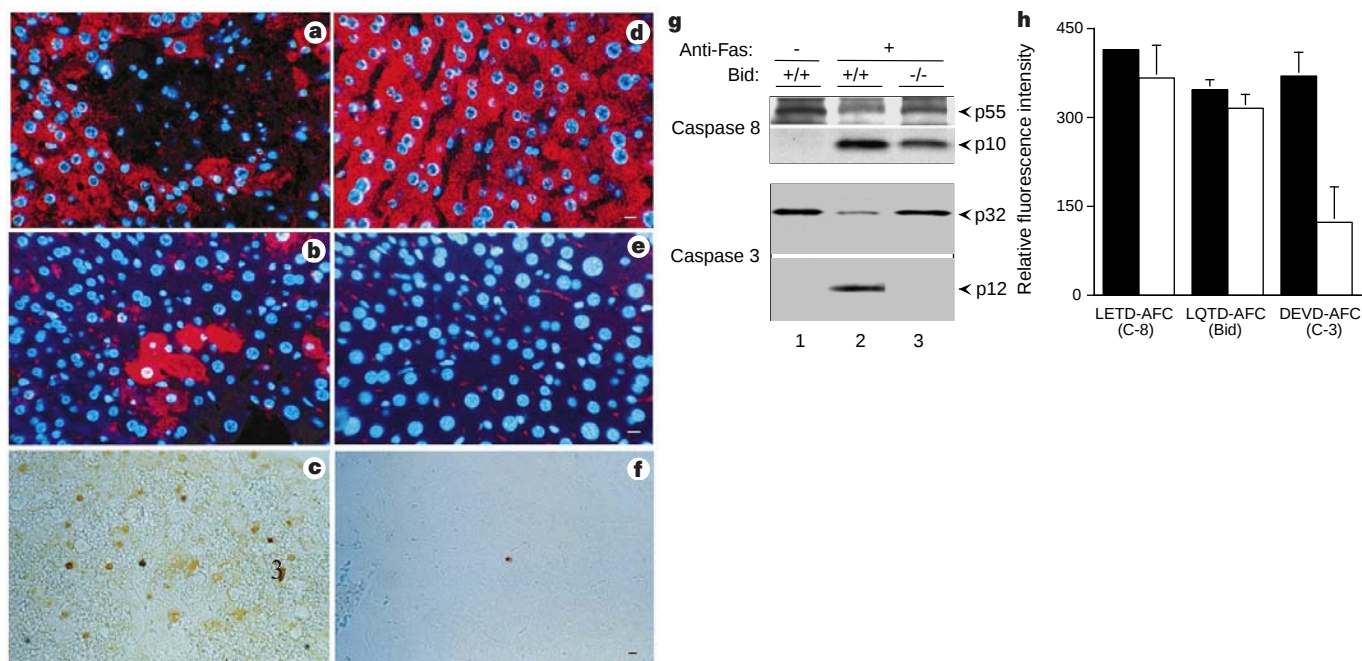


Figure 3 Lack of cytochrome *c* release, caspase activation or DNA fragmentation in unaffected *Bid*^{-/-} hepatocytes in response to anti-Fas antibody. **a–f**, Assessment of cytochrome *c* (**a**, **d**), activation of caspases 3 and 7, as detected by CM1 (ref. 30) (**b**, **e**) and DNA fragmentation by TUNEL (**c**, **f**) in hepatocytes of WT (**a–c**) and unaffected *Bid*^{-/-} mice (**d–f**). Scale bar, 25 μ m. Typically, onset of apoptosis in WT hepatocytes is accompanied by loss of cytochrome *c* staining (**a**). **g**, Analysis of the activation of caspases 8 and 3 in hepatocytes by immunoblot 2 h after injection of anti-Fas antibody. The p10 caspase-8 and p12 caspase-3 fragments

represent the activated proteases. **h**, Caspase activity in S100 fractions prepared from hepatocytes 2 h after injection with anti-Fas antibody for WT (black bars) and unaffected *Bid*^{-/-} (white bars). Caspase activity was measured by release of the AFC fluorochrome from peptide substrates selective for caspase 8 (LETD), caspases 3 and 7 (DEVD), or the Bid site (LQTD). Data are mean $\pm$ s.d. of two mice from one of four representative experiments. The difference in the activity of caspases 3 and 7 between WT and *Bid*^{-/-} hepatocytes is statistically significant ($P < 0.05$).

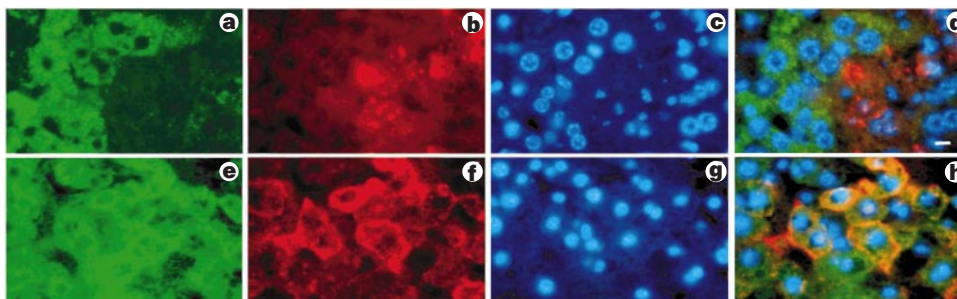


Figure 4 Altered pattern of apoptosis in affected *Bid*^{-/-} hepatocytes 2 h after injection of anti-Fas antibody. Comparison of **a**, **e**, cytochrome *c* status; **b**, **f**, activation of caspases 3 and 7 by staining with CM1 antibody³⁰; **c**, **g**, bisbenzimidazole nuclear morphology in the same liver section of *Bid*^{+/-} (**a–d**) or affected *Bid*^{-/-} mice

(**e–h**); **d**, **h** show three-colour immunohistochemical staining for cytochrome *c* (green), activated caspases 3 and 7 by CM1 (red) and nuclear morphology (blue). Scale bar, 25 μ m.

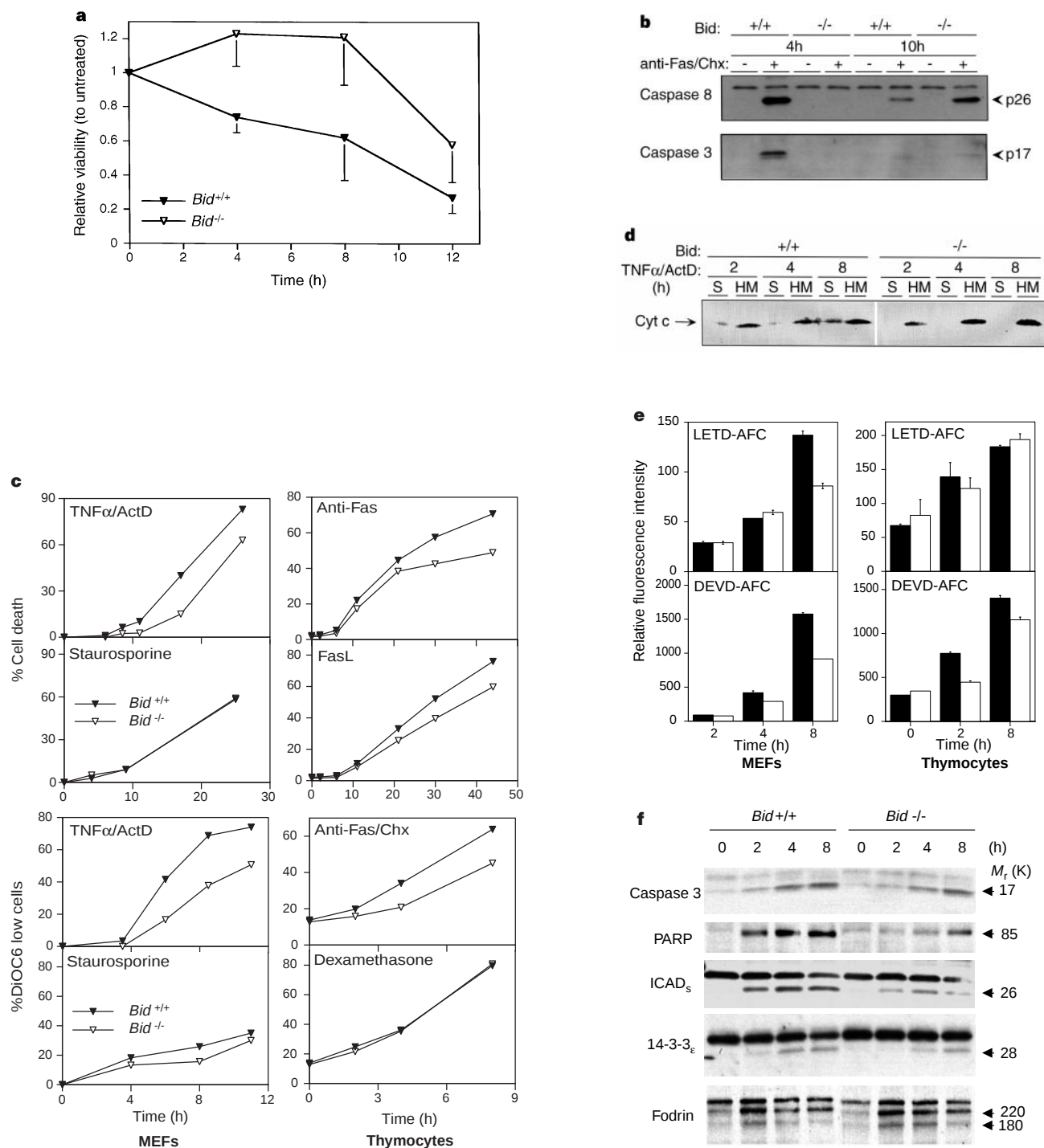


Figure 5 Altered apoptotic program in anti-Fas-treated cultured hepatocytes and thymocytes or TNF α -treated embryonic fibroblasts (MEFs) from $Bid^{-/-}$ mice. **a**, Viability of cultured hepatocytes from $Bid^{+/+}$ (filled triangles) or $Bid^{-/-}$ (triangles) mice treated *in vitro* with anti-Fas antibody/cycloheximide. The spontaneous death rate of untreated hepatocytes is subtracted and the relative viability plotted. **b**, Activation of caspase 8 (p26 intermediate fragment) and caspase 3 (p17 fragment) at 4 and 10 h after treatment of hepatocytes with anti-Fas/cycloheximide (Chx). **c**, Cell viability of MEFs treated with TNF α /ActD versus staurosporine, and thymocytes treated with anti-Fas antibody or recombinant FasL. Loss of mitochondrial transmembrane potential, as assessed by the percentage of cells with low DiOC6 fluorescence, after treatment of WT (filled symbols) or $Bid^{-/-}$ (triangles) MEFs with TNF α /ActD or staurosporine and thymocytes with anti-Fas Ab/Chx or dexamethasone. Data shown for MEFs and thymocytes are from one of three representative experiments. **d**, Analysis of cytochrome *c* release in $Bid^{+/+}$ versus $-/-$ MEFs after treatment with TNF α /ActD for 2, 4 or 8 h. The heavy

membrane (HM) fraction is enriched for mitochondria (confirmed by anti-cytochrome oxidase antibody); the soluble S100 fraction (S) is the cytosolic component. Fractions were screened by western blot with anti-cytochrome *c* antibody. **e**, Time course of caspase activity in WT (black bars) or $Bid^{-/-}$ (white bars) MEFs and thymocytes after treatment with TNF α /ActD or anti-Fas antibody/Chx, respectively. Data for MEFs and thymocytes are mean $\pm$ s.d. from duplicate samples from one of three representative experiments. Values for $Bid^{+/+}$ versus $-/-$ MEFs at 8 h after treatment were significant for caspases 3 and 7 ($P < 0.0005$) and caspase 8 ($P < 0.005$), whereas thymocytes varied significantly for caspases 3 and 7 ($P < 0.01$), but not for caspase 8 ($P > 0.05$). **f**, Altered pattern of cleavage of caspase substrates within $Bid^{-/-}$ versus $Bid^{+/+}$ thymocytes after treatment with anti-Fas antibody. Immunoblot detection of the cleavage of the cytoskeletal substrate fodrin was comparable in $-/-$ and $+/+$ cells, whereas cleavage of ICADs and PARP, which participate in the nuclear programme, was delayed and diminished in $Bid^{-/-}$ cells as was the 14-3-3 substrate.

Other *Bid*^{-/-} cells died, although they underwent a moderated pathway of apoptosis which defined a Bid-dependent component that affects mitochondrial function and cytochrome *c* release, the recruitment of effector caspases, selection of substrates and nuclear damage. The differential cleavage of substrates may reflect the amount and restricted distribution of effector caspases in the absence of a Bid-induced cytochrome *c*/Apaf-1/caspase-9 amplification loop²³, needed to generate a more effective and widely distributed activation of effector caspases. This may account in part for the decreased cellular injury and improved survival of *Bid*-deficient cells and mice. Retention of cytochrome *c* and the sustained transmembrane potential in null cells also implicate mitochondria in the Bid pathway. Death stimuli that do not use the TNFR1/Fas receptor are not much affected by Bid deficiency. Our *Bid*-deficient mice did not undergo any alteration in developmental cell death, as noted in knockouts of caspase 8 (ref. 24), caspase 9 (refs 25, 26) or Apaf-1 (refs 27, 28). The Bid-dependent pathway also shows cell-type specificity, suggesting that pro-apoptotic Bcl-2 members²⁹ may act as selective links between specific upstream signals and downstream death effectors. Our results from null mice indicate that Bid may have evolved to trigger a mitochondrial amplification loop to ensure the death of cell types having limited access to initiator or effector caspases and/or in response to weak death-receptor signals. □

Methods

Generation of *Bid*^{-/-} mice. A P1 murine genomic clone of 129/SvJ origin containing the *Bid* locus was identified. Restriction mapping and DNA-sequence analysis of subcloned fragments revealed that the murine *Bid* gene contains at least 5 exons and 4 introns spanning a region of at least 7.5 kb (ref. 9). A 5-kb 3' fragment and a 2.1-kb 5' fragment were cloned into a gene-replacement vector pNTK. The deleted 4.2-kb central region, replaced by a neomycin-resistance gene (*neo*), eliminates part of exon 1 containing the initiation methionine, exon 2, and part of exon 3, including the BH3 domain. The targeting vector was transfected into RW4 ES cells and neomycin-resistant clones were selected. Southern-blot analysis using a 3' external probe recognizes a 9.5-kb *Hind*III wild-type fragment and an 8.2-kb *Hind*III homologous recombinant fragment. Three homologous recombinant RW4 clones were identified and two were injected into C57BL/6 blastocysts. Six chimaeric males from these two ES clones transmitted to the germ line. Mouse genotypes were determined by a three-primer PCR approach and confirmed by Southern blotting of genomic DNA isolated from tail biopsies. Western blots of lysates prepared from thymocytes and splenocytes were separated by 12.5% SDS-PAGE. Antibodies used were a polyclonal rabbit anti-murine Bid, a monoclonal anti-murine Bcl-2 (3F11), and a polyclonal anti-murine Bax (651) antibody. Secondary goat antibodies were conjugated with horseradish peroxidase (CALTAG). Blots were developed by ECL (Amersham).

Animal and cell-culture experiments. Young adult mice were injected intravenously with anti-Fas antibody (clone Jo2; Pharmingen) at a dose of 0.25 µg g⁻¹. Animals were either observed over 48–72 h for mortality or killed at matched time points for liver histology or biochemical studies. Thymocytes were prepared from *Bid*^{+/+} or *Bid*^{-/-} mice and cultured with control buffer, 1 µg ml⁻¹ anti-Fas antibody (clone Jo2), or 100 nM recombinant FasL plus 30 µg ml⁻¹ cycloheximide (Chx; Sigma) or 1 µM dexamethasone. Murine embryonic fibroblasts were prepared and treated with 1 ng ml⁻¹ TNF-α (Sigma) plus 2 µg ml⁻¹ actinomycin D (ActD; Sigma) or 4 µM staurosporine. Livers were perfused with calcium and magnesium-free Hank's solution, followed by L15 medium containing 5 mM CaCl₂ and 0.5 mg ml⁻¹ collagenase H. Liver parenchymal cells were isolated from the dissected liver by low-speed centrifugation and cultured in William's E medium containing 10% FCS. Following attachment for 2–3 h, hepatocytes were treated with anti-Fas antibody (0.5–1 µg ml⁻¹) plus Chx (10 µg ml⁻¹). Cells were analysed for viability, mitochondrial transmembrane potential ($\Delta\Psi_M$) and caspase activation.

Histology and immunohistochemical staining. Livers were weighed, cut into small (<1.0 cm) pieces and fixed overnight at 4 °C in 4% paraformaldehyde in PBS, Bouin's solution, or methanolic Carnoy's fixative. The tissue was then

dehydrated, paraffin-embedded and cut at 4-µm thickness. Haematoxylin-and-eosin stained sections were used for histopathological evaluation of hepatic injury. Slides were scored by a pathologist blinded to the genotype of the mice and the experimental conditions. Livers were graded on a scale of zero to three. Grade 0 corresponds to normal hepatic histology; 1, rare (<1 per 40× field) apoptotic nuclei; 2, moderate numbers of (>1 per 40× field) apoptotic nuclei, with no or only rare focal haemorrhages; 3, severe hepatic injury, with frequent apoptotic nuclei and marked haemorrhage. TUNEL staining was done on paraformaldehyde-fixed sections.

Immunohistochemical staining was done using tyramide signal amplification (NEN Life Science Products)³⁰. Cytochrome *c* immunoreactivity was detected with mouse anti-cytochrome *c* antibodies (Pharmingen), and activated caspase-3 and 7 immunoreactivity with an affinity-purified rabbit polyclonal antiserum (CM1). This antiserum immunohistochemically recognizes both activated caspases 3 and 7, but not their zymogenic forms³⁰. Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies and direct TSA, using either fluorescein or cyanine-3-conjugated tyramine. For dual cytochrome *c* and CM1 immunolabelling, sequential TSA immunodetection was performed. Fluorescently labelled sections were incubated with bisbenzimidazole (Hoechst 33258) before coverslipping to stain cell nuclei. Fluorescence was recorded with a Zeiss-Axioskop epifluorescence microscope and MC-100 camera system.

Caspase activation. Caspase activity was assessed by the cleavage of site-selected tetrapeptide reporter substrates with the relative specificity of LETD-AFC (caspase 8), LQTD-AFC (caspase-8 cleavage site in Bid), DEVD-AFC (caspases 3 and 7) (Enzyme Systems Products). Lysates of hepatocytes or thymocytes containing 25–50 µg of protein were incubated at 37 °C with 50 µM peptide substrates for 30 min. Fluorescence generated by the release of the fluorogenic group AFC (aminotrifluoromethyl-coumarin) upon cleavage by caspases, was detected at the excitation wavelength of 400 nm and an emission wavelength of 505 nm with an FL500 microplate fluorescence reader (Bio-Tek). The signal strength representative of caspase activity was corrected for background and standardized to equivalent protein. Activated fragments of caspases 8 and 3 and other caspase substrates were detected by western blot. Liver, thymocyte and MEF lysates were prepared in 10 mM Tris-HCl, pH 7.2, 142.5 mM KCl, 5 mM MgCl₂, 1 mM EDTA and 0.25% NP-40 with protease inhibitors (0.2 mM PMSF, 0.1% aprotinin, 1 µg ml⁻¹ pepstatin, 1 µg ml⁻¹ leupeptin). Equal amounts of protein were size-fractionated by 8% or 15% SDS-PAGE, transferred to a PVDF membrane and immunoblots developed using polyclonal anti-Bid, anti-caspase-3, anti-caspase-8, anti-14-3-3 (Santa Cruz), anti-ICAD, anti-PARP (Chemicon) or anti-fodrin (Chemicon) antibodies. Secondary goat antibody was conjugated with HRP (CALTAG) and western blots were developed with ECL (Amersham).

Analysis of cell viability and mitochondrial transmembrane potentials. Thymocytes or MEFs were assessed for viability after treatment by propidium iodide exclusion and/or staining with 0.5 µg ml⁻¹ annexin V in the presence of calcium for 30 min and analysis by flow cytometry. Viable cells were gated based on low annexin V staining and high forward light scatter. Cells were incubated with 40 nM of DiOC6 (ref. 2) (3,3'-dihexyloxacarbocynine iodide; Molecular Probes) for 30 min to determine mitochondrial transmembrane potential. Cells with DiOC6 staining as a reflection of decreased $\Delta\Psi_M$ were detected by flow cytometry.

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1. Luo, X., Budihardjo, I., Zou, H., Slaughter, C. & Wang, X. Bid, a Bcl-2 interacting protein, mediates cytochrome *c* release from mitochondria in response to activation of cell surface death receptors. *Cell* **94**, 481–490 (1998).
2. Li, H., Zhu, H., Xu, C. J. & Yuan, J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94**, 491–501 (1998).
3. Gross, A. *et al.* Caspase-cleaved BID targets mitochondria and is required for cytochrome *c* release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. *J. Biol. Chem.* **274**, 1156–1163 (1999).
4. Han, Z., Bhalla, K., Pantazis, P., Hendrickson, E. A. & Wyche, J. H. Cif (cytochrome *c* efflux-inducing factor) activity is regulated by Bcl-2 and caspases and correlates with the activation of Bid. *Mol. Cell. Biol.* **19**, 1381–1389 (1999).
5. Sun, X. M. *et al.* Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. *J. Biol. Chem.* **274**, 5053–5060 (1999).
6. Chou, J., Li, H., Salvesen, G., Yuan, J. & Wagner, G. Solution structure of BID, an intracellular amplifier of apoptotic signaling. *Cell* **96**, 615–624 (1999).
7. McDonnell, J., Fushman, D., Millman, C., Korsmeyer, S. & Cowburn, D. Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonist and antagonists. *Cell* **96**, 625–634 (1999).

8. Wang, K., Yin, X. M., Chao, D. T., Millman, C. L. & Korsmeyer, S. J. BID: a novel BH3 domain-only death agonist. *Genes Dev.* **10**, 2859–2869 (1996).
9. Wang, K. *et al.* BID, a proapoptotic BCL-2 family member, is localized to mouse chromosome 6 and human chromosome 22q11. *Genomics* **53**, 235–238 (1998).
10. Tartaglia, L. A., Ayres, T. M., Wong, G. H. W. & Goeddel, D. V. A novel domain within the 55 kd TNF receptor signals cell death. *Cell* **74**, 845–853 (1993).
11. Nagata, S. Apoptosis: telling cells their time is up. *Curr. Biol.* **6**, 1241–1243 (1996).
12. Boldin, M. P., Goncharov, M., Goltsev, Y. V. & Wallach, D. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell* **85**, 803–815 (1996).
13. Muzio, M. *et al.* FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/CD95) death-inducing signaling complex. *Cell* **85**, 817–827 (1996).
14. Ogasawara, J. *et al.* Lethal effect of the anti-Fas antibody in mice. *Nature* **364**, 806–809 (1993).
15. Thornberry, N. A. *et al.* A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J. Biol. Chem.* **272**, 17907–17911 (1997).
16. Liu, X., Kim, C. N., Yang, J., Jemmerson, R. & Wang, X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* **86**, 147–157 (1996).
17. Jemmerson, R. *et al.* A conformational change in cytochrome c of apoptotic and necrotic cells is detected by monoclonal antibody binding and mimicked by association of the native antigen with synthetic phospholipid vesicles. *Biochemistry* **38**, 3599–3609 (1999).
18. Scaffidi, C. *et al.* Two CD95 (APO-1/Fas) signaling pathways. *EMBO J.* **17**, 1675–1687 (1998).
19. Xuesong, L., Zou, H., Slaughter, C. & Wang, X. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* **89**, 175–184 (1997).
20. Enari, M. *et al.* A caspase-activated DNase that degrades DNA during apoptosis and its inhibitor ICAD. *Nature* **391**, 43–50 (1998).
21. Lacronique, V. *et al.* Bcl-2 protects from lethal hepatic apoptosis induced by an anti-Fas antibody in mice. *Nature Med.* **2**, 80–86 (1996).
22. Rodriguez, I. *et al.* A bcl-2 transgene expressed in hepatocytes protects mice from fulminant liver destruction but not from rapid death induced by anti-Fas antibody injection. *J. Exp. Med.* **183**, 1031–1036 (1996).
23. Li, P. *et al.* Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**, 479–489 (1997).
24. Varfolomeev, E. E. *et al.* Targeted disruption of the mouse caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1 and DR3 and is lethal prenatally. *Immunity* **9**, 267–276 (1998).
25. Kuida, K. *et al.* Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell* **94**, 325–337 (1998).
26. Hakem, R. *et al.* Differential requirement for caspase 9 in apoptotic pathways *in vivo*. *Cell* **94**, 339–352 (1998).
27. Cecconi, F., Alvarez-Bolado, G., Meyer, B. I., Roth, K. A. & Gruss, P. Apaf1 (Ced-4 homolog) regulates programmed cell death in mammalian development. *Cell* **94**, 727–737 (1998).
28. Yoshida, H. *et al.* Apaf1 is required for mitochondrial pathways of apoptosis and brain development. *Cell* **94**, 739–750 (1998).
29. Adams, J. M. & Cory, S. The Bcl-2 family: arbiters of cell survival. *Science* **281**, 1322–1326 (1998).
30. Srinivasan, A. *et al.* *In situ* immunodetection of activated caspase-3 in apoptotic neurons in the developing nervous system. *Cell Death Differ.* **5**, 1004–1016 (1998).

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Activation of the ERK/MAPK pathway by an isoform of rap1GAP associated with G α_i

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Many receptors for neuropeptides and hormones are coupled with the heterotrimeric G $\beta\gamma$ protein, which activates the p42/44 mitogen-activated protein kinase (ERK/MAPK) cascade through both the α - and $\beta\gamma$ -subunits of G $\beta\gamma$ (refs 1–3). The $\beta\gamma$ -subunit activates the ERK/MAPK cascade through tyrosine kinase^{4–6}. Constitutively active G α_{i2} (gip2) isolated from adrenal and ovarian tumours^{7,8} transforms Rat-1 fibroblasts and also activates the ERK/MAPK cascade by an unknown mechanism^{9,10}. The ERK/MAPK pathway is activated by Ras, and is inhibited when the low-molecular-mass

GTP-binding protein Rap1 antagonizes Ras function¹¹. Here we show that a novel isoform of Rap1 GTPase-activating protein, called rap1GAPII, binds specifically to the α -subunits of the G $\beta\gamma$ family of heterotrimeric G-proteins. Stimulation of the G $\beta\gamma$ -coupled m2-muscarinic receptor translocates rap1GAPII from the cytosol to the membrane and decreases the amount of GTP-bound Rap1. This decrease in GTP-bound Rap1 activates ERK/MAPK. Thus, the α -subunit of G $\beta\gamma$ activates the Ras-ERK/MAPK mitogenic pathway by membrane recruitment of rap1GAPII and reduction of GTP-bound Rap1.

To elucidate the signalling pathways coupled to G α_i in mammalian cells, we searched for proteins that interact with G α_i using the yeast two-hybrid system. We used rat G α_{i1} as a bait and screened complementary DNA libraries derived from Jurkat cells and human fetal brain. Sixteen independent positive clones were isolated from 10⁶ colonies, and four contained cDNAs for rap1GAP¹². In the initial screens, the isolated cDNAs of these clones were found to be fused to the activator domain of GAL4 in the putative 5' non-coding region of the rap1GAP cDNA, which proved to be essential for G α_i interaction. We sought new isoforms of rap1GAP in which the putative 5' non-coding region of rap1GAP was translated from a previously unrecognized translation-initiation codon. Rescreening of cDNA libraries for an isoform of rap1GAP in which this putative untranslated region is translated and expressed led to identification of nine new independent clones for rap1GAP cDNA, four of which contained a 5' extension and a previously unrecognized ATG codon (Fig. 1a in Supplementary Information). We designated the protein encoded by this cDNA as rap1GAPII to differentiate it from the prototype rap1GAP. Rap1GAPII cDNA encodes an additional 31 amino acids before the first Met of the prototype rap1GAP.

Expression of rap1GAPII messenger RNA was examined by polymerase chain reaction with reverse transcription (RT-PCR), which revealed rap1GAPII expression in heart, liver, kidney and cerebrum (Fig. 2a in Supplementary Information). A primer set common to all rap1GAPs detected mRNA in all of the tissues examined. The presence of the splice variants of rap1GAP cDNA was confirmed by ribonuclease protection assay. In Jurkat T cells, from which rap1GAPII cDNA was originally isolated, we detected only the fragment specific to rap1GAPII cDNA, whereas in 143B osteosarcoma cells fragments for both rap1GAPII and the prototype rap1GAP were detected (Fig. 2b in Supplementary Information).

We investigated the specificity of the rap1GAPII–G α_i interaction using a yeast two-hybrid system (Fig. 1 in Supplementary Information). β -Gal and His activity under the regulation of the GAL4–rap1GAPII fusion protein was detected when the α -subunits of the G $\beta\gamma$ family of proteins, G α_{i1} , G α_{i2} , G α_{i3} and G α_{o} , were used as baits, but not with G α_s , G α_q or G α_{13} . Removal of the first 31 amino acids, which are unique to rap1GAPII, completely abolished the interaction of rap1GAPII with G α_{i1} , indicating that rap1GAPII but not the prototype rap1GAP interacts with G α_i . Residues 26–31 of the amino-terminal portion of rap1GAPII were critical to the interaction.

We have tested the specificity of GAP activity of rap1GAPII to H-Ras, R-Ras and Rap1 in 293T cells. For measurement of the GAP activity of rap1GAPII, GTP-bound forms of H-Ras and Rap1 were accumulated by co-expression of mSos1 and C3G, respectively. Rap1GAPII stimulated the GTPase activity of Rap1, but not of H-Ras or R-Ras (Fig. 2c in Supplementary Information). As little as a one-hundredth molar ratio of rap1GAPII to C3G was sufficient to diminish the GTP-bound Rap1 to the basal level. These data demonstrate the high specificity and potent activity of rap1GAPII for Rap1 *in vivo*.

Binding of rap1GAPII to G α_{i1} was confirmed with 293T human kidney-cell lysates expressing G α_{i1} and rap1GAPII proteins tagged with glutathione S-transferase (GST). GST–rap1GAPII specifically associated with the GTPase-deficient G α_{i1} QL more than with the

wild-type $G\alpha_{i1}$ (Fig. 1a). The prototype GST-rap1GAP did not bind to either form of $G\alpha_{i1}$. Similar results were obtained using the wild-type and GTPase-deficient $G\alpha_{i2}$ or Myc-tagged rap1GAPII. To show further that the interaction between rap1GAPII and $G\alpha_i$ was mediated by amino-acids 26–31, we performed precipitation experiments using GST fusion proteins. Figure 1b shows that amino-acids 26–31 were necessary for association of GST-rap1GAPII and $G\alpha_i$.

To test the interaction of rap1GAPII and $G\alpha_i$ in cells expressing rap1GAPII endogenously, we used Jurkat cells for co-immunoprecipitation studies (Fig. 1c). $G\alpha_{i2}$ was co-immunoprecipitated with the anti-rap1GAP immunoprecipitate in Jurkat cells stably transfected with the $G\alpha_{i2}$ QL expression vector, and to a lesser extent in cells transfected with the $G\alpha_{i2}$ WT (wild-type) expression vector. A band that co-migrated with $G\alpha_{i2}$ was also detected in the lane of the anti-rap1GAP immunoprecipitate from non-transfected Jurkat cells, indicating the binding of rap1GAPII to the endogenous $G\alpha_i$

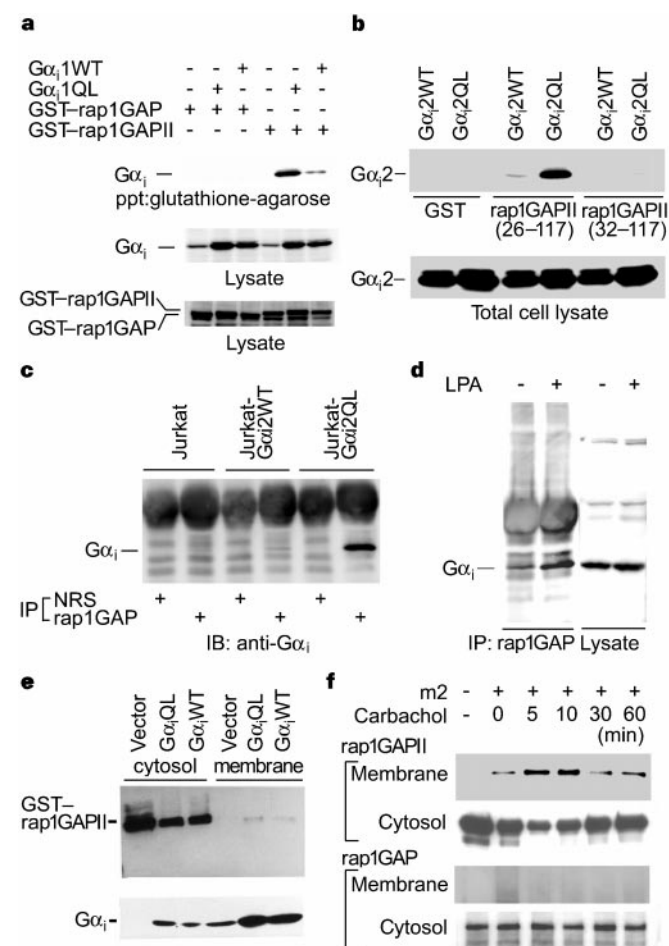


Figure 1 Binding of rap1GAPII to $G\alpha_i$. **a**, 293T cells were transfected with the combination of expression vectors indicated. The total lysates and glutathione-bound fraction were analysed by immunoblotting. **b**, Cell lysate of 293T cells transfected with plasmids indicated was precipitated with bacterially expressed GST-tagged rap1GAPII proteins indicated at the bottom. **c**, Total cell lysates of Jurkat, Jurkat- $G\alpha_{i2}$ WT, and Jurkat- $G\alpha_{i2}$ QL cells were immunoprecipitated with normal rabbit serum (NRS) or anti-rap1GAP antibody (rap1GAP). **d**, NIH3T3-loxP-rap1GAPII cells infected with Cre-expressing adenovirus were serum-starved and stimulated with LPA. **e**, 293T cells were transfected with the plasmids indicated. Localization of GST-rap1GAPII and $G\alpha_i$ was analysed by immunoblotting. **f**, 293T cells transfected with (+) or without (–) m2-muscarinic receptor expression vector were serum-starved and stimulated with 1 mM Carbachol for the indicated period.

protein(s). Binding of rap1GAPII to endogenous $G\alpha_i$ was further demonstrated by the use of 3T3-loxP-rap1GAPII cells, in which rap1GAPII is induced upon infection of Cre-expressing adenovirus. The amount of $G\alpha_i$ in the anti-rap1GAPII immunoprecipitates increased upon stimulation by lysophosphatidic acid (LPA), which activates $G\alpha_i$ -coupled receptors (Fig. 1d).

Many of the guanine nucleotide exchange factors (GEF) and GTPase-activating proteins of Ras-family proteins are regulated by membrane recruitment from the cytosol¹³. We therefore examined the effect of $G\alpha_i$ expression on the subcellular localization of rap1GAPII (Fig. 1e). Rap1GAPII expressed in 293T cells was present mostly in the cytosol fraction without $G\alpha_{i1}$. Co-expression of $G\alpha_{i1}$, which is localized mainly in the membrane fraction, translocated rap1GAPII to the membrane. In 293T cells expressing the m2-muscarinic receptor, stimulation by Carbachol, an agonist of the muscarinic receptor, rapidly translocated rap1GAPII, but not rap1GAP, into the membrane fraction (Fig. 1f).

We next investigated the effects of $G\alpha_i$ -coupled receptor stimulation on the GTPase activity of Rap1, by measuring the amount of GTP-bound Rap1. Carbachol stimulation of 293T cells expressing the m2-muscarinic receptor and rap1GAPII rapidly decreased the amount of GTP-bound Rap1 (Fig. 2a). This reduction in GTP-bound Rap1 was abolished by overexpression of the dominant-negative rap1GAPII that associates with $G\alpha_i$ but lacks the GTPase-activating domain.

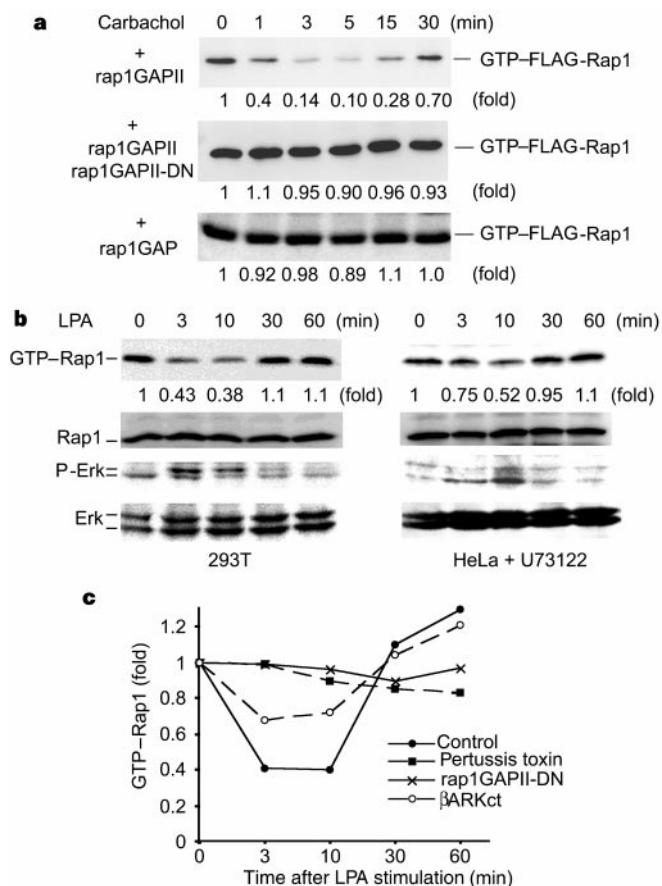


Figure 2 $G\alpha_i$ -dependent downregulation of Rap1. **a**, 293T cells expressing FLAG-tagged Rap1 and the proteins indicated on the left were stimulated with 1 mM carbachol. **b**, 293T and HeLa cells were serum-starved and stimulated with LPA. HeLa cells were pretreated with U73122 before stimulation. Endogenous GTP-bound Rap1 and proteins indicated on the left were detected by immunoblotting. Activated ERK/MAPK is depicted as P-Erk. **c**, 293T cells were untreated, or incubated with pertussis toxin, or transfected with the expression vector for the dominant negative mutant of rap1GAPII or β ARK.

We further examined whether activation of the G_{α_i} -coupled receptor reduced GTP-bound Rap1 under more physiological conditions. 293T cells were serum-starved for 48 h and stimulated with lysophosphatidic acid (LPA). As shown in Fig. 2b, the amount of GTP-bound Rap1 was decreased rapidly by LPA. The decrease coincided with the appearance of the activated form of ERK/MAPK. A similar, but prolonged, decrease in GTP-bound Rap1 was also observed in NIH 3T3 cells upon LPA stimulation. However, when HeLa cells were stimulated with LPA, the amount of GTP-bound Rap1 was increased slightly, as reported previously¹⁴. Under this condition, the activation of CalDAG-GEFI, which is a Rap1-specific GEF regulated by Ca^{2+} and diacylglycerol¹⁵, may dominate the rap1GAPII-induced decrease in GTP-bound Rap1, because LPA activates phospholipase C (PLC)- β through G_q -coupled receptor(s). Hence, in the next experiment, we pretreated HeLa cells with U73122, an inhibitor of PLC, and then stimulated them with LPA.

LPA stimulation rapidly decreased the GTP-bound Rap1 in HeLa cells, as shown in Fig. 2b.

The role of the α - and $\beta\gamma$ -subunits of G_i in the LPA-dependent decrease in GTP-bound Rap1 was examined using pertussis toxin and dominant-negative mutants of rap1GAPII and β -adrenergic receptor kinase (β ARK)¹⁶. As shown in Fig. 2c, pertussis toxin and rap1GAPII-DN, but not β ARK-ct, inhibited the LPA-dependent decrease in GTP-bound Rap1, indicating that the effect is mediated by G_{α_i} .

Rap1 suppresses many Ras-dependent signalling cascades, including cellular transformation and ERK/MAPK activation by LPA and epidermal growth factor (EGF)^{11,17,18}. Thus, we next examined whether the suppression of Rap1 by rap1GAPII could enhance Ras-dependent signalling cascades even with basal Ras activity. Activation of ERK/MAPK was examined in 293T cells in the presence of rap1GAPII. Expression of rap1GAPII activated ERK/MAPK (Fig. 3a). Expression of rap1GAP also activated ERK/MAPK, albeit to a lesser extent. Because expression of rap1GAPII did not increase GTP-bound Ras, this result supports the proposal that reduction of GTP-bound Rap1 is sufficient to activate ERK/MAPK in 293T cells. When we co-expressed a GTPase-deficient mutant of Rap1, Rap1V12, rap1GAPII did not activate ERK/MAPK. Induction of rap1GAPII in NIH 3T3-loxP-rap1GAP cells also activated ERK/MAPK, as detected by an anti-active ERK/MAPK antibody (Fig. 3b).

To explore the specific activation of ERK/MAPK by rap1GAPII or rap1GAP, we stimulated 293T cells expressing the m2-muscarinic receptor (Fig. 3c, d). In this experiment, expression of rap1GAPII and rap1GAP was reduced so as not to stimulate ERK/MAPK before carbachol stimulation. Carbachol-dependent activation of ERK/MAPK was significantly enhanced by rap1GAPII, whereas EGF-dependent ERK/MAPK activation was not enhanced by rap1GAPII. The prototype rap1GAP did not enhance the m2-dependent activation of ERK/MAPK.

Finally, we compared the contribution of the G_{α_i} -dependent and $\beta\gamma$ -subunit-dependent pathways to the m2-dependent activation of Elk-1, a transcription factor stimulated directly by ERK/MAPK, using dominant-negative mutants of rap1GAPII and β ARK¹⁶. The dominant-negative β ARK mutant efficiently inhibited both m1- and m2-dependent activation of Elk-1 (Fig. 3e). The dominant-negative rap1GAPII mutant, however, inhibited Elk-1 activation by the m2-muscarinic receptor, but not by the m1-muscarinic receptor, indicating that rap1GAPII participated only in G_{α_i} -dependent Elk-1 activation. Because dominant-negative mutants of both rap1GAPII and β ARK inhibited m2-dependent Elk-1 activation, it appears that the activation of Ras through $\beta\gamma$ -subunits and inactivation of Rap1 through G_{α_i} synergistically transduce signals to the ERK/MAPK cascade.

The role of Rap1 in the regulation of ERK/MAPK activity is still controversial¹⁹. Rap1 was originally isolated as a suppressor of Ras-dependent transformation¹⁷ and was subsequently shown to inhibit Ras-dependent ERK/MAPK activation¹¹. Consistent with this, Rap1-dependent inhibition of ERK/MAPK causes T-cell anergy²⁰. However, in the nervous system or in *Drosophila melanogaster*, Rap1 seems to activate ERK/MAPK^{21–23}. In another report, activation of Rap1 does not correlate with either suppression or activation of ERK/MAPK¹⁴. From these reports, transient activation of Rap1 does not seem to compete with the ERK/MAPK pathway; however, a high basal level of Rap1 in cells transfected with plasmids or in the anergic state of T cells may suppress ERK/MAPK. Our observation that a decrease in GTP-bound Rap1 by rap1GAPII activates ERK/MAPK without Ras activation supports this view. Rap1 localizes mainly in the endocytic and lysosomal vesicles²⁴, although it is believed to compete with Ras at the plasma membrane. Thus, most of the external stimuli used in the previous studies may not activate Rap1 at the plasma membrane and, therefore, may not inhibit the Ras-dependent activation of ERK/MAPK.

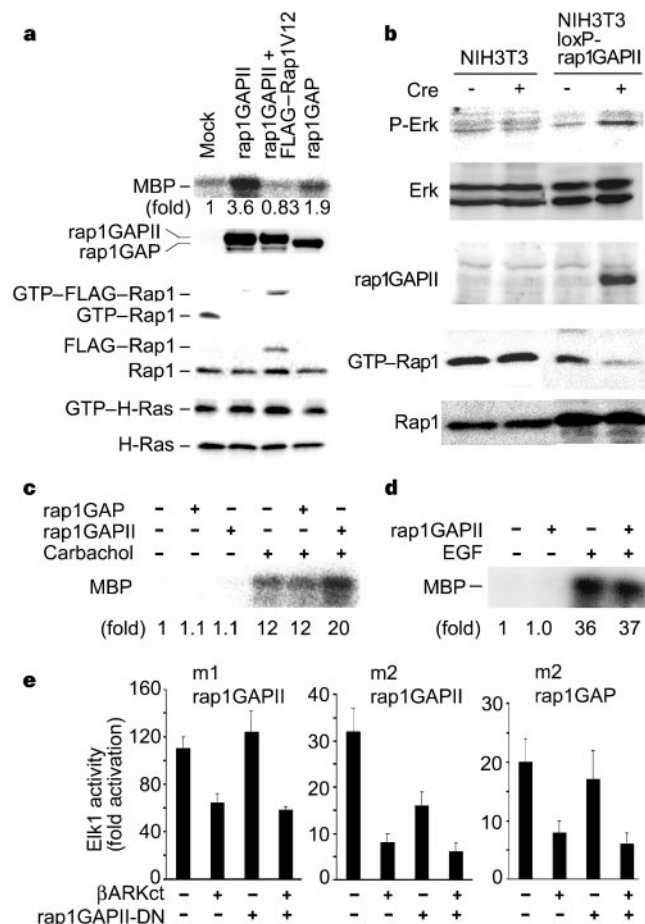


Figure 3 Activation of ERK/MAPK and Elk1 transcription factor by rap1GAPII. **a**, 293T cells were transfected with the expression vectors for GST-ERK2 and the proteins indicated on the top. Myelin basic protein (MBP) was used as a substrate for ERK/MAPK. **b**, NIH3T3 or NIH3T3-loxP-rap1GAPII cells were infected with Cre-expressing adenovirus. Activated ERK/MAPK, depicted as P-Erk, and proteins indicated on the left were detected by immunoblotting. **c**, **d**, 293T cells were transfected without or with pEBG-rap1GAPII or pEPB-rap1GAP in combination with expression vectors encoding human m2-muscarinic receptor or EGF receptor. **e**, 293T cells were transfected with expression vectors indicated at the top and bottom and stimulated with Carbachol. m1, pEF-hm1 (m1 muscarinic receptor); m2, pEF-hm2 (m2 muscarinic receptor); rap1GAPII, pEBG-rap1GAPII; rap1GAP, pEPB-rap1GAP; rap1GAPII-DN, pEBG-rap1GAPII (1–148) dominant-negative mutant; β ARKct, pRK5- β ARKct dominant-negative mutant.

In conclusion, we have identified a new pathway for $G\alpha_i$ -mediated activation of the ERK/MAPK pathway and mitogenesis. This pathway provides the first example, to our knowledge, of $G\alpha_i$ -subunit-mediated crosstalk of the ERK/MAPK pathway and complements $\beta\gamma$ -mediated activation of mitogenesis. □

Methods

For detailed methods, see Supplementary Information. Briefly, the initial cDNA of rap1GAPII was isolated by yeast two-hybrid screening using pGBT9- $G\alpha_{i1}$, which encoded the entire coding region of $G\alpha_{i1}$. Another lambda phage cDNA library was screened by probing with rap1GAP cDNA to look for the longer isoform of rap1GAP in which the 5' untranslated region of rap1GAP is translated and expressed. To detect binding of rap1GAPII to $G\alpha_i$ in the cultured cells, rap1GAP was immunoprecipitated from the lysates of Jurkat or NIH3T3 cells and analysed by immunoblotting with anti- $G\alpha_i$ antibody. The level of GTP-bound Rap1 or Ras was measured as described²⁵. Activation of ERK/MAPK was measured either by *in vitro* kinase assay or by the use of an anti-phosphorylated-ERK/MAPK antibody. Activation of Elk1 transcription factor was measured with a PathDetect kit (Stratagene).

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- Howe, L. R. & Marshall, C. J. Lysophosphatidic acid stimulates mitogen-activated protein kinase activation via a G-protein-coupled pathway requiring $p21^{ras}$ and $p74^{raf-1}$. *J. Biol. Chem.* **268**, 20717–20720 (1993).
- Alblas, J. et al. Gi-mediated activation of the p21ras-mitogen-activated protein kinase pathway by alpha 2-adrenergic receptors expressed in fibroblasts. *J. Biol. Chem.* **268**, 22235–22238 (1993).
- Wintz, S. et al. Involvement of Ras and Raf in the Gi-coupled acetylcholine muscarinic m2 receptor activation of mitogen-activated protein (MAP) kinase kinase and MAP kinase. *J. Biol. Chem.* **268**, 19196–19199 (1993).
- Crespo, P., Xu, N. Z., Simonds, W. F. & Gutkind, J. S. Ras-dependent activation of MAP kinase pathway mediated by G-protein $\beta\gamma$ subunits. *Nature* **369**, 418–420 (1994).
- Koch, W. J., Hawes, B. E., Allen, L. F. & Lefkowitz, R. J. Direct evidence that Gi-coupled receptor stimulation of mitogen-activated protein kinase is mediated by G- $\beta\gamma$ activation of p21ras. *Proc. Natl Acad. Sci. USA* **91**, 12706–12710 (1994).
- van Biesen, T. et al. Receptor-tyrosine-kinase- and G $\beta\gamma$ -mediated MAP kinase activation by a common signalling pathway. *Nature* **376**, 781–784 (1995).
- Lyons, J. et al. Two G protein oncogenes in human endocrine tumors. *Science* **249**, 655–659 (1990).
- Wong, Y. H. et al. Mutant alpha subunits of Gi2 inhibit cyclic AMP accumulation. *Nature* **351**, 63–65 (1991).
- Pace, A. M., Wong, Y. H. & Bourne, H. R. A mutant alpha subunit of Gi2 induces neoplastic transformation of Rat-1 cells. *Proc. Natl Acad. Sci. USA* **88**, 7031–7035 (1991).
- Gupta, S. K., Gallego, C., Johnson, G. L. & Heasley, L. E. MAP kinase is constitutively activated in gip2 and src transformed rat1a fibroblasts. *J. Biol. Chem.* **267**, 7987–7990 (1992).
- Cook, S. J., Rubinfeld, B., Albert, I. & McCormick, F. RapV12 antagonizes Ras-dependent activation of ERK1 and ERK2 by LPA and EGF in Rat-1 fibroblasts. *EMBO J.* **12**, 3475–3485 (1993).
- Rubinfeld, B. et al. Molecular cloning of a GTPase activating protein specific for the Krev-1 protein p21^{ras}. *Cell* **65**, 1033–1042 (1991).
- McCormick, F. Ras-related proteins in signal transduction and growth control. *Mol. Reprod. Dev.* **42**, 500–506 (1995).
- Zwartkruis, F. J. et al. Extracellular signal-regulated activation of Rap1 fails to interfere in Ras effector signalling. *EMBO J.* **17**, 5905–5912 (1998).
- Kawasaki, H. et al. A Rap guanine nucleotide exchange factor enriched highly in the basal ganglia. *Proc. Natl Acad. Sci. USA* **95**, 13278–13283 (1998).
- Della Rocca, G. J. et al. Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptors. Convergence of Gi- and Gq-mediated pathways on calcium/calmodulin, Pyk2, and Src kinase. *J. Biol. Chem.* **272**, 19125–19132 (1997).
- Kitayama, H. et al. A ras-related gene with transformation suppressor activity. *Cell* **56**, 77–84 (1989).
- Okada, S. et al. Insulin regulates the dynamic balance between Ras and Rap1 signaling by coordinating the assembly states of the Grb2-SOS and CrkII-C3G complexes. *EMBO J.* **17**, 2554–2565 (1998).
- Bos, J. L. All in the family? New insights and questions regarding interconnectivity of Ras, Rap1 and Raf. *EMBO J.* **17**, 6776–6782 (1998).
- Boussiotis, V. A. et al. Maintenance of human T cell anergy: blocking of IL-2 gene transcription by activated Rap1. *Science* **278**, 124–128 (1997).
- Vossler, M. R. et al. cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell* **89**, 73–82 (1997).
- York, R. D. et al. Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. *Nature* **392**, 622–626 (1998).
- Ishimaru, S. et al. Activation of the Drosophila C3G leads to cell fate changes and overproliferation during development, mediated by the Ras-MAPK pathway and RAP1. *EMBO J.* **18**, 145–155 (1999).
- Pizon, V. et al. Association of Rap1a and Rap1b proteins with late endocytic/phagocytic compartments and Rap2a with the Golgi complex. *J. Cell Sci.* **107**, 1661–1670 (1994).
- Franke, B., Akkerman, J. W. N. & Bos, J. L. Rapid Ca^{2+} -mediated activation of Rap1 in human platelets. *EMBO J.* **15**, 252–259 (1997).

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Extracellular sodium regulates airway ciliary motility by inhibiting a P2X receptor

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The mucociliary system is responsible for clearing inhaled particles and pathogens from the airways. This important task is performed by the beating of cilia and the consequent movement of mucus from the lungs to the upper airways^{1,2}. Because ciliary motility is enhanced by elevated intracellular calcium concentrations, inhibition of calcium influx could lead to disease by jeopardizing mucociliary clearance. Several hormones and neurotransmitters stimulate ciliary motility, one of the most potent of which is extracellular ATP (ATP_o)¹, which acts by releasing calcium ions from internal stores and by activating calcium influx^{3–5}. Here we show that, in airway ciliated cells, extracellular sodium ions (Na_o^+) specifically and competitively inhibit an ATP_o -gated channel that is permeable to calcium ions, and thereby attenuate ATP_o -induced ciliary motility. Our findings point to a physiological role for Na_o^+ in ciliary function, and indicates that

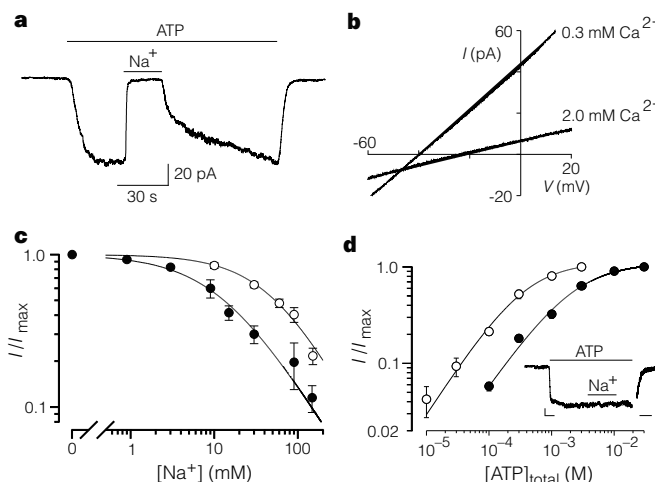


Figure 1 Na_o^+ competitively inhibits the current activated by ATP_o . **a**, Whole-cell current recording showing activation of an inward current by ATP_o (200 μM) that was reversibly inhibited by replacing the extracellular Cs⁺ (151 mM) with Na⁺. **b**, Net ATP_o -activated current responses to voltage ramps (0.1 V s⁻¹) in extracellular NMDG-Cl solutions containing either 0.3 or 2.0 mM CaCl₂. Raising extracellular Ca²⁺ shifted the reversal potential of the current from -40 to -24 mV, indicating that Ca²⁺ is permeable. **c**, Dose-response relationships for inhibition by Na_o^+ of the current activated by ATP_o in extracellular solutions containing 1.58 mM (filled circles) or 0.5 (open circles) mM CaCl₂ (16 and 50 μM ATP_o, respectively). IC₅₀ and (n) were 13 mM (0.92) and 53 mM (1.04), respectively. Each data point is the average (±s.e.m.) of 5–7 experiments. **d**, Dose-response relationships for ATP_o without (open circles) and with (filled circles) 15 mM Na_o⁺. Each data point is the average (±s.e.m.) of 5–14 experiments. EC₅₀, n and a are 0.37 mM, 0.92 and 1.1 without Na_o⁺ and 2.20 mM, 1.04 and 1.1 with Na_o⁺, respectively. The current activated by a saturating concentration of ATP_o (30 mM) was not inhibited by 15 mM Na_o⁺ (inset); current and time calibrations are 5 pA and 5 s (left) and 30 s (right).

mucociliary clearance might be improved in respiratory disorders such as chronic bronchitis and cystic fibrosis by decreasing the sodium concentration of the airway surface fluid in which the cilia are bathed.

Rabbit airway ciliated cells express a novel ATP-gated cation-selective channel ($P2X_{cilia}$) that is strongly attenuated by extracellular divalent cations⁶. We now report the unexpected regulation of $P2X_{cilia}$ channels by Na^+ . Figure 1a shows a whole-cell current trace recorded at a holding potential of -40 mV. In CsCl solutions, ATP_o activated an inward current through $P2X_{cilia}$ channels that was reversibly inhibited by replacing the extracellular Cs^+ with Na^+ ($n = 19$). As $P2X_{cilia}$ channels are permeable to Ca^{2+} (Fig. 1b; $n = 28$), inhibition of $P2X_{cilia}$ channels by Na^+ could have profound effects on the regulation of mucociliary clearance by ATP_o^{3–5}. Consequently, we examined the nature of the inhibition of $P2X_{cilia}$ channels by sodium ions.

A dose–response relationship for the channel inhibition by Na^+ was constructed from experiments of the type shown in Fig. 1a (filled circles in Fig. 1c). The data are well described by a Hill equation with a single binding site for Na^+ and an IC_{50} of 13 mM (continuous line through filled circles in Fig. 1c). We found that the inhibitory effect of Na^+ depends on the concentration of ATP_o. Increasing the effective concentration of ATP_o shifts the dose–response curve for Na^+ to higher concentrations ($IC_{50} = 53$ mM; open circles in Fig. 1c). This finding indicates that Na^+ may competitively inhibit the effects of ATP_o. If so, the dose–response relationship for ATP_o should be shifted to higher concentrations by Na^+ . Indeed, the EC_{50} for ATP_o was shifted from 0.37 to 2.2 mM by 15 mM Na^+ (Fig. 1d), whereas the response to a saturating concentration of ATP_o was not inhibited by Na^+ (Fig. 1d, inset). These results show that the degree of inhibition by Na^+ depends on interplay between the effective concentrations of ATP_o and Na^+ . In addition, as the effects of Na^+ could be overcome by raising the concentration of ATP_o, we conclude that the ability of Na^+ to inhibit $P2X_{cilia}$ channels does not result from blocking of the ion permeation pathway by Na^+ .

The inhibitory effect of Na^+ could arise from a direct effect of Na^+ on $P2X_{cilia}$ channels, or from an indirect effect of Na^+ involving

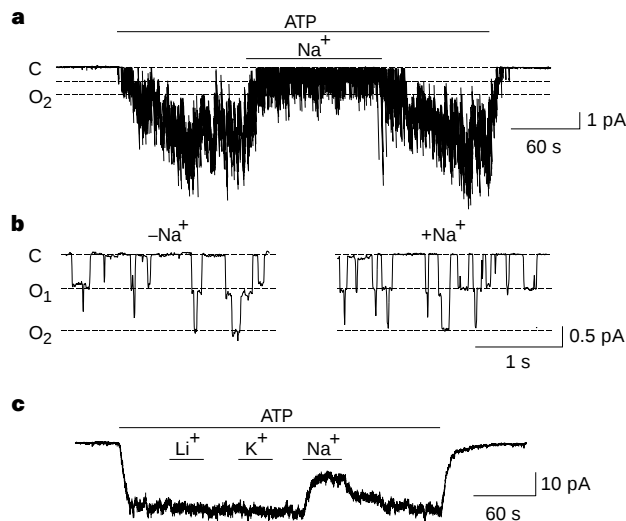


Figure 2 Na^+ directly and specifically inhibits $P2X_{cilia}$ channels. **a**, Continuous current trace recorded at -120 mV from an outside-out membrane patch. ATP_o ($40 \mu M$) activated an inward current that was partially and reversibly inhibited by 15 mM Na^+ . **b**, Excerpts from the current trace shown in **a** before ($-Na^+$) and during ($+Na^+$) inhibition by 15 mM Na^+ . The dashed lines in **a** and **b** indicate the current levels for a single channel opening (O_1), for the simultaneous opening of two channels (O_2) and for no channel activation (C). **c**, The whole-cell current activated by ATP_o ($200 \mu M$) was not inhibited by 15 mM Li^+ or K^+ .

phosphorylation or dephosphorylation of the channels, or involving a G-protein-coupled process. To determine whether $P2X_{cilia}$ channels are directly modulated by Na^+ , we examined the effects of Na^+ on the currents activated by ATP_o in excised outside-out patches of membrane. Consistent with the inhibition of whole-cell currents by Na^+ shown in Fig. 1, the ensemble single-channel currents activated by ATP_o were attenuated by 15 mM Na^+ (Fig. 2a; $n = 16$). However, the single-channel current amplitudes were the same with and without Na^+ (Fig. 2b; $n = 6$). Thus, Na^+ directly modulates $P2X_{cilia}$ channels, either by reducing the number of active channels or by reducing the activity of each channel. Regardless of the molecular mechanism of inhibition, the effect of Na^+ is highly specific, as neither smaller (Li^+) nor larger (K^+) alkali metal cations (15 mM) inhibited the conductance activated by ATP_o (Fig. 2c; $n = 14$).

Na^+ also inhibited ATP_o-activated conductances in airway ciliated cells from rat ($n = 6$), pig ($n = 7$) and human ($n = 6$) (data not

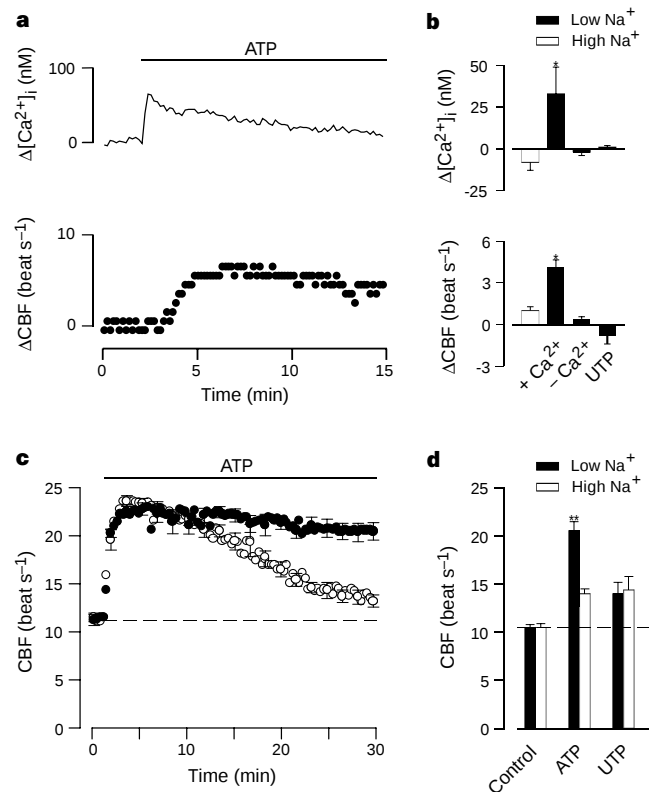


Figure 3 Na^+ attenuates ATP_o-induced ciliary activity by inhibiting $P2X_{cilia}$ channels. **a**, Simultaneous measurement of $[Ca^{2+}]_i$ (top) and CBF (bottom) from a cell bathed in low (10 mM) Na^+ , in which the $P2Y$ pathway was suppressed (see Methods). ATP_o ($100 \mu M$) enhanced $[Ca^{2+}]_i$ and CBF. **b**, Summary of the change in $[Ca^{2+}]_i$ (top) and CBF (bottom) induced by either ATP_o ($100 \mu M$) or UTP_o ($100 \mu M$) on cells in which the $P2Y$ pathway was suppressed. In high (140 mM) Na^+ , ATP_o had no effect. ATP_o increased both $[Ca^{2+}]_i$ and CBF in low Na^+ when the extracellular solution contained 0.5 mM $CaCl_2$ ($+Ca^{2+}$), but not when the extracellular solution contained $0.5 \mu M$ $CaCl_2$ ($-Ca^{2+}$). UTP_o had no effect in low Na^+ with 0.5 mM $CaCl_2$ in the extracellular solution (UTP). Measurements were made 5 min after the addition of ATP or UTP. The number of experiments of CBF and ($[Ca^{2+}]_i$) measurements in each condition are: ATP_o in high Na^+ , 8 and (5); $+Ca^{2+}$, 10 and (7); $-Ca^{2+}$, 8 and (5); UTP_o, 9 and (5). Values represent mean $\pm$ s.e.m. $*P < 0.02$. **c**, Average CBF recorded in 140 mM ($n = 10$; open circles) or 10 mM ($n = 9$; filled circles) extracellular $NaCl$ in control cells (where the $P2Y$ pathway is functional). CBF enhancement induced by ATP_o ($100 \mu M$) was attenuated by Na^+ . Every tenth error bar is shown. **d**, Summary of the change in CBF in control cells induced by either ATP_o ($100 \mu M$) or UTP_o ($100 \mu M$) in low or high Na^+ . Measurements were made 25 min after the addition of ATP or UTP. The numbers of experiments in each condition in low and (high) Na^+ are control, 26 and (30); ATP_o, 9 and (10); UTP_o, 8 and (8). Values represent mean $\pm$ s.e.m. $**P < 0.0001$.

shown). As $P2X_{cilial}$ channels are permeable to Ca^{2+} (Fig. 1b), and Ca^{2+} influx is required to maintain elevated ciliary activity in the presence of ATP_o , we postulated that ciliary activation by ATP_o could be modulated by Na_o^+ . Hence, we investigated whether activation of $P2X_{cilial}$ channels by ATP_o can elevate the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and enhance the ciliary beating frequency (CBF).

In high Na_o^+ , ATP_o enhances CBF primarily by activating a G-protein-coupled (P2Y) receptor that is sensitive to both ATP and UTP⁵. To study the effects of $P2X_{cilial}$ -channel activation on CBF, we suppressed the P2Y-mediated pathway by depleting the intracellular Ca^{2+} stores with thapsigargin and by inhibiting phospholipase C with U-71322. We measured basal and stimulated ciliary activity and $[Ca^{2+}]_i$ from single ciliated cells in explants of rabbit airway epithelium in extracellular solutions containing either low (10 mM) or high (140 mM) concentrations of Na^+ . The ionic strength of the solution was kept constant by equimolar substitution of NaCl by NMDG-Cl. In the absence of stimulation, CBF was the same with low and high Na_o^+ , indicating that Na_o^+ does not have a general non-specific effect on ciliary motility (Fig. 3d; control). When the P2Y pathway was suppressed, ATP_o (100 μ M) failed to elevate $[Ca^{2+}]_i$ or to enhance ciliary activity when Na_o^+ was high (Fig. 3b). In contrast, in low Na_o^+ , ATP_o elevated $[Ca^{2+}]_i$ and enhanced CBF, provided that the extracellular solution contained Ca^{2+} (Fig. 3a, b). As extracellular UTP (UTP_o) does not activate $P2X_{cilial}$ channels⁶, we predicted that UTP_o would not elevate $[Ca^{2+}]_i$ or stimulate the cilia in low Na_o^+ . Indeed, UTP_o (100 μ M) had no effect (Fig. 3b). These results show that Ca^{2+} influx through $P2X_{cilial}$ channels can enhance ciliary activity.

To determine whether the P2Y pathway is also modulated by Na_o^+ , we compared ciliary activation by UTP_o with that by ATP_o in cells in which the P2Y pathway was not suppressed. Ciliary activation by UTP_o (100 μ M) was the same with low and high Na_o^+ , indicating that the P2Y pathway that is activated by either UTP or ATP⁵ is not modulated by Na_o^+ (Fig. 3d). In contrast, Na_o^+ suppressed the response to ATP_o , which activates both the P2Y pathway and $P2X_{cilial}$ channels. In low (but not high) Na_o^+ , CBF remained at a high level of activation throughout the exposure to ATP_o (Fig. 3c). After 25 min with ATP_o , the CBF was 20.6 ± 0.9 beats per s in low Na_o^+ , whereas in high Na_o^+ , the CBF was only 14.0 ± 0.5 beats per s (Fig. 3d). The initial rise in CBF induced by ATP_o was not affected by Na_o^+ , indicating that the P2Y pathway can maximally stimulate the cilia. This stimulation, however, is transient. These results show that ciliary activation by ATP_o is modulated by Na_o^+ and indicate that this modulation is mediated by $P2X_{cilial}$ channels.

Our findings uncover a new physiological mechanism for regulating ciliary function: the activation of cilia by ATP_o can be attenuated by Na_o^+ . Modulation of beat frequency by Na_o^+ could have a profound effect on the efficiency of mucociliary clearance, as the energy transferred by the cilia to the mucus blanket is proportional to the square of the CBF⁷. Reports that Na^+ modulates human β -defensin, the peptide antibiotic expressed by the surface epithelia in lung^{8–10}, and now that Na^+ regulates ciliary motility by inhibiting $P2X_{cilial}$ channels, indicate that modulation of protein function by Na_o^+ may be a fundamental physiological mechanism of airway function.

In the proximal airways, the Na^+ content of the airway surface fluid (ASF) appears to be normally lower than in plasma^{11–13}, and has been reported to be either unchanged^{12–14} or elevated^{11,15} in cystic fibrosis. In contrast, little is known of the composition of the ASF in the distal airways¹⁶. If Na^+ is elevated in the distal airways in disorders such as cystic fibrosis, then one of the early events leading to impaired mucociliary clearance may be a reduced ability of ATP_o to regulate ciliary motility. In any event, preventing inhibition by Na_o^+ of ciliary activity is a potential new route for improving the clearance of mucus. As hypertonic solutions, per se, improve the rheological properties of mucus^{17,18}, and may increase the volume of the ASF¹⁴, we propose that inhalation of a hypertonic aerosol that

does not contain Na^+ could improve mucociliary clearance more than the currently used hypertonic saline aerosol^{19–23}. □

Methods

Cells and solutions. Single rabbit, rat and human airway ciliated cells were enzymatically isolated as described⁶. Human airway ciliated cells were isolated from surgically excised nasal polyps. Solutions were made with highly purified water (NANOpure, Barnstead), using chemicals of analytical grade. The standard extracellular solution contained (mM): 151 CsCl, 1.58 $CaCl_2$, 5 TES, 10 D-glucose, 1,3,4-diaminopyridine, adjusted to pH 7.4 with CsOH (295–300 mOsmol). The standard intracellular (pipette) solution contained (mM): 155 CsCl, 5 TES, 0.1 EGTA, 1 Mg-ATP, 0.1 Na_2 GTP, 0.037 $CaCl_2$, adjusted to pH 7.2 with CsOH (285–290 mOsmol). In most outside-out patch experiments, the pipette solution contained 0.2 mM GDP- β S in place of GTP and 1 mM EDTA in place of EGTA, and both Mg-ATP and $CaCl_2$ were omitted. Solutions containing alkali cations other than Cs^+ were prepared by equimolar substitution of Cs^+ by the desired cation. Solutions containing ATP or other nucleotides were prepared fresh each day and the pH was readjusted. As the current through $P2X_{cilial}$ channels is strongly attenuated by divalent cations⁶, in some experiments the effective concentration of ATP was increased by lowering extracellular $CaCl_2$ from 1.58 to 0.5 mM (ref. 6). When the extracellular $CaCl_2$ concentration was lowered from 0.5 mM to 0.5 μ M, the effective concentration of ATP was kept the same by the addition of 0.28 mM $MgCl_2$. To maintain the same ionic driving force in the different K_2 ATP solutions, ATP dose–response curves were generated using KCl in place of CsCl in the extracellular solution, and 2 mM KCl were omitted for each mM of K_2 ATP. In these experiments, volume-sensitive Cl^- currents activated by the change in the osmolarity were eliminated by setting the Cl^- reversal potential to the holding potential (–40 mV). Thus, intracellular Cl^- was set to 30 mM by replacing 125 mM of the intracellular Cl^- with MES[–]. In the NaCl-containing extracellular solution, 15 mM KCl were replaced by 15 mM NaCl.

Electrophysiological recordings. Membrane currents were recorded using the standard whole-cell (at –40 mV) and outside-out patch configurations of the patch-clamp technique as described⁶. Recordings were initiated at least 5 min after establishing the whole-cell configuration to allow full equilibration of the cytosol with the pipette solution. Membrane currents were recorded under voltage-clamp (Axopatch 200A) and were low-pass filtered using an 8-pole Bessel filter (Frequency Devices). The sampling frequency was set to at least two times the corner frequency of the low-pass filter. We used Sigma Plot 2.0 scientific software (Jandel Scientific) for nonlinear curve fitting. Measurements were at room temperature (18–22 °C).

Dose–response curves. When generating the ATP_o dose–response curves, the currents were normalized to account for rundown as described⁶. The data were fitted with the equation $I/I_{max} = a([ATP_o]^n / ([ATP_o]^n + EC_{50}^n))$, where I is the current activated by a given concentration of ATP_o , I_{max} is the maximal current activated by ATP_o , EC_{50} is the concentration of ATP_o ($[ATP_o]$) yielding a current half the maximum, a is a normalization factor and n is the Hill coefficient. The Na_o^+ inhibition data were fitted with the equation $I/I_{max} = 1 - (1 / (1 + (IC_{50}/[Na_o^+])^n))$, where I is the current activated by ATP_o at a given concentration of Na_o^+ , I_{max} is the current activated by ATP_o in the absence of Na_o^+ , IC_{50} is the concentration of Na_o^+ ($[Na_o^+]$) that inhibits the current by 50% and n is the Hill coefficient.

CBF and $[Ca^{2+}]_i$ measurements. The preparation of monolayer explants from rabbit airway epithelium, CBF measurements using the photometric method and $[Ca^{2+}]_i$ measurements with the fluorescent indicator fura-2 were as described^{4,5}. Measurements were performed on 7–14-day-old explants. We measured CBF and $[Ca^{2+}]_i$ at 37 °C, as ciliary activity is highly temperature sensitive. CBF (at a resolution of ± 1 beats per s) was averaged over 10 s. Before each experiment, the explants were placed in the test solution for at least 15 min because of possible transient effects of Na_o^+ on $[Ca^{2+}]_i$ ²⁴. In most experiments, the explants were continuously superfused with the desired extracellular solution to prevent a possible reduction in the concentration of ATP_o due to degradation by electronucleotidases. In some experiments the P2Y pathway was suppressed by emptying the calcium stores by pre-incubating the cells for 40 min with thapsigargin (1 μ M) and by inhibiting the phospholipase C pathway by pre-incubating the cells for 5–7 minutes with U-71322 (2 μ M). Groups were compared using Student's *t*-test.

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1. Wanner, A., Salathé, M. & O'Riordan, T. G. Mucociliary clearance in the airways. *Am. J. Respir. Crit. Care Med.* **154**, 1868–1902 (1996).
2. Matsui, H., Randell, S. H., Peretti, S. W., Davis, C. W. & Boucher, R. C. Coordinated clearance of periciliary liquid and mucus from airway surfaces. *J. Clin. Invest.* **102**, 1125–1131 (1998).
3. Paradiso, A. M., Mason, S. J., Lazarowski, E. R. & Boucher, R. C. Membrane-restricted regulation of Ca^{2+} release and influx in polarized epithelia. *Nature* **377**, 643–646 (1995).
4. Kornegreen, A. & Priel, Z. Purinergic stimulation of rabbit ciliated airway epithelia: control by multiple calcium sources. *J. Physiol. (Lond.)* **497**, 53–66 (1996).
5. Uzlauer, N. & Priel, Z. Interplay between the NO pathway and elevated $[\text{Ca}^{2+}]_i$ enhances ciliary activity in rabbit trachea. *J. Physiol. (Lond.)* **516**, 179–190 (1999).
6. Kornegreen, A., Ma, W., Priel, Z. & Silberberg, S. D. Extracellular ATP directly gates a cation-selective channel in rabbit airway ciliated epithelial cells. *J. Physiol. (Lond.)* **508**, 703–720 (1998).
7. Silberberg, A. On mucociliary transport. *Biorheology* **27**, 295–307 (1990).
8. Smith, J. J., Travis, S. M., Greenberg, E. P. & Welsh, M. J. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell* **85**, 229–236 (1996).
9. Goldman, M. J. *et al.* Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell* **88**, 553–560 (1997).
10. Bals, R. *et al.* Human beta-defensin 2 is a salt-sensitive peptide antibiotic expressed in human lung. *J. Clin. Invest.* **102**, 874–880 (1998).
11. Joris, L., Dab, I. & Quinton, P. M. Elemental composition of human airway surface fluid in healthy and diseased airways. *Am. Rev. Respir. Dis.* **148**, 1633–1637 (1993).
12. Knowles, M. R. *et al.* Ion composition of airway surface liquid of patients with cystic fibrosis as compared with normal and disease-control subjects. *J. Clin. Invest.* **100**, 2588–2595 (1997).
13. Hull, J., Skinner, W., Robertson, C. & Phelan, P. Elemental content of airway surface liquid from infants with cystic fibrosis. *Am. J. Respir. Crit. Care Med.* **157**, 10–14 (1998).
14. Matsui, H. *et al.* Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airway disease. *Cell* **95**, 1005–1015 (1999).
15. Zabner, J., Smith, J. J., Karp, P. H., Widdicombe, J. H. & Welsh, M. J. Loss of CFTR chloride channels alters salt absorption by cystic fibrosis airway epithelia *in vitro*. *Mol. Cell* **2**, 397–403 (1998).
16. Wine, J. J. The genesis of cystic fibrosis lung disease. *J. Clin. Invest.* **103**, 309–312 (1999).
17. Daviskas, E. *et al.* Inhalation of dry-powder mannitol increases mucociliary clearance. *Eur. Respir. J.* **10**, 2449–2454 (1997).
18. Wills, P. J., Hall, R. L., Chan, W. M. & Cole, P. J. Sodium chloride increases the ciliary transportability of cystic fibrosis and bronchiectasis sputum on the mucus-depleted bovine trachea. *J. Clin. Invest.* **99**, 9–13 (1997).
19. Pavia, D., Thomson, M. L. & Clarke, S. W. Enhanced clearance of secretions from the human lung after the administration of hypertonic saline aerosol. *Am. Rev. Respir. Dis.* **117**, 199–203 (1978).
20. Daviskas, E. *et al.* Inhalation of hypertonic saline aerosol enhances mucociliary clearance in asthmatic and healthy subjects. *Eur. Respir. J.* **9**, 725–732 (1996).
21. Eng, P. A. *et al.* Short-term efficacy of ultrasonically nebulized hypertonic saline in cystic fibrosis. *Pediatr. Pulmonol.* **21**, 77–83 (1996).
22. Robinson, M. *et al.* Effect of hypertonic saline, alimemazine, and cough on mucociliary clearance in patients with cystic fibrosis. *Am. J. Respir. Crit. Care Med.* **153**, 1503–1509 (1996).
23. Robinson, M. *et al.* Effect of increasing doses of hypertonic saline on mucociliary clearance in patients with cystic fibrosis. *Thorax* **52**, 900–903 (1997).
24. Boitano, S., Woodruff, M. L. & Dirksen, E. R. Reduction of extracellular Na^+ causes a release of Ca^{2+} from internal stores in airway epithelial cells. *Am. J. Physiol.* **272**, L1189–L1197 (1997).

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A new pathway for polyketide synthesis in microorganisms

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Chalcone synthases, which biosynthesize chalcones (the starting materials for many flavonoids^{1,2}), have been believed to be specific to plants. However, the *rppA* gene from the Gram-positive, soil-living filamentous bacterium *Streptomyces griseus* encodes a 372-amino-acid protein that shows significant similarity to chalcone synthases³. Several *rppA*-like genes are known, but their functions and catalytic properties have not been described. Here we show that a homodimer of RppA catalyses polyketide synthesis: it selects malonyl-coenzyme-A as the starter, carries out four successive extensions and releases the resulting pentaketide to cyclize to 1,3,6,8-tetrahydroxynaphthalene (THN). Site-directed mutagenesis revealed that, as in other chalcone synthases^{4,5}, a cysteine residue is essential for enzyme activity. Disruption of the chromosomal *rppA* gene in *S. griseus* abolished melanin production in hyphae, resulting in 'albino' mycelium. THN was readily oxidized to form 2,5,7-trihydroxy-1,4-naphthoquinone (flavio-

lin), which then randomly polymerized to form various coloured compounds. THN formed by RppA appears to be an intermediate in the biosynthetic pathways for not only melanins but also various secondary metabolites containing a naphthoquinone ring. Therefore, RppA is a chalcone-synthase-related synthase that synthesizes polyketides and is found in the *Streptomyces* and other bacteria.

rppA encoding a chalcone synthase (CHS)-like protein was cloned from *S. griseus* as a gene that conferred red-brown pigment production on *Streptomyces* spp. and *Escherichia coli*³. We found that *rppA* actually encoded a protein with relative molecular mass 40,000 ($M_r = 40K$) (the nucleotide sequence reported in ref. 3 has been corrected; EMBL, DDBJ and GeneBank databases, accession no. AB018074). Because RppA showed 30% identity in amino-acid sequence to plant-specific CHSs, we produced RppA in *E. coli* and examined its enzymatic activity to elucidate its catalytic function. The recombinant RppA had a histidine tag at its amino terminus for convenience in purification. RppA purified from the soluble fraction using a histidine-bind resin gave a single protein band of $M_r = 42K$ on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). The native enzyme appeared to be a dimer as it had an apparent M_r of $\sim 90K$, as determined by gel-filtration-column chromatography (data not shown).

Plant-specific CHSs use coenzyme-A (CoA)-esters from the phenylpropanoid pathway and malonyl-CoA to synthesize chalcones. Because of sequence similarity between RppA and plant CHSs, we first incubated *p*-coumaroyl-CoA and [2-¹⁴C]malonyl-CoA in the presence of RppA. Analysis of the reaction mixture by thin-layer chromatography (TLC) showed two radioactive compounds with an R_f value different from that of the expected product, naringenin (data not shown). Unexpectedly, further examination by reverse-phase high-performance liquid chromatography (HPLC) showed that incubation of [1-¹⁴C]malonyl-CoA alone with RppA yielded the same radioactive product (Fig. 2a, d). Prolonged incubation of the reaction mixture resulted in the gradual disappearance of peak 1 with a concomitant increase in the amount and radioactivity of peak 2. As described below, peak 2 represents flaviolin that is derived non-enzymatically from peak 1. We also determined the activity of a mutant enzyme (RppA/C138S) with an amino-acid replacement at Cys 138 with serine, as amino-acid alignment of RppA with other CHSs^{4,5} predicted that Cys 138 served as the active site of the condensation reaction. RppA/C138S showed no enzyme activity (Fig. 2c, f), indicating that Cys 138 of RppA has the same function in catalysis as in other plant CHSs.

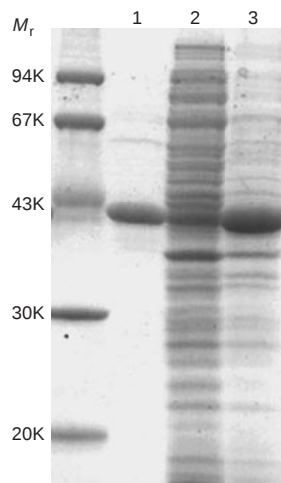


Figure 1 SDS-PAGE analysis of RppA. Lane 1, RppA purified by affinity chromatography with a His-bind resin; lane 2, soluble fraction; and lane 3, insoluble fraction. RppA was produced in both soluble and insoluble fractions. Each lane contains proteins at appropriate amounts.

Liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (LC–APCIMS) analysis of the products indicated that the primary product was a pentaketide, as it gave m/z at 193 and 191 in positive and negative mode MS, respectively. Multistage MS fragmentation patterns were all consistent with those of THN, an established melanin precursor in fungi. Direct comparison of the LC–APCIMS patterns of the primary product and an authentic sample from a fungus *Colletotrichum lagenarium*⁶ revealed that the product was THN. A minor product (peak 2) at 21 min on LC co-eluted with flaviolin, a non-enzymatic oxidation product of THN which was further identified by similar LC–APCIMS comparison with an authentic specimen from *C. lagenarium*⁶. Considering a possible pathway from malonyl-CoA to THN, we examined the possibility that acetyl-CoA resulting from decarboxylation of malonyl-CoA served as a starter, as is the case for the plant CHS-related enzyme pyrone synthase (GCHS2)⁷ and a fungal 6-methylsalicylic acid synthase with no similarity to CHSs⁸. The presence of [1-¹⁴C]acetyl-CoA in the reaction mixture containing RppA and malonyl-CoA did not give radioactive products (Fig. 2b, e) or affect the synthesis rate of THN. We propose the RppA-catalysed THN biosynthetic pathway shown in Fig. 3a. RppA uses malonyl-CoA as the starter, catalyses chain elongation through successive condensation of C2 units to form the pentaketide and releases the resulting ketide to cyclize to THN. The final ring closure to THN involves the removal of a carboxyl group of the malonyl-CoA used as the starter substrate. Thus, RppA is a CHS-related polyketide synthase. Because of the instability of THN, it may be oxidized to form flaviolin, which then polymerizes to form various coloured compounds. This is consistent with the observation that the red-brown pigments produced by *E. coli* containing *rppA* on a plasmid were a mixture of compounds that were readily precipitated with ethanol³. Microorganisms possess type I polyketide synthases, consisting of giant multifunctional proteins and type II enzymes, consisting of several separate, largely monofunctional proteins; both polyketide synthases, as in fatty acid biosynthesis, sequentially assemble CoA-derivatives of carboxylic acids into aliphatic carbon chains^{9–11}.

A computer-aided search predicted that the gene clusters for biosynthesis of 2,4-diacetylphloroglucinol in *Pseudomonas fluorescens*¹² and of chloroeremomycin (a vancomycin derivative) in *Amycolatopsis orientalis*¹³ contain a gene homologous to *rppA*. On the basis of the catalytic function of RppA, 2,4-diacetylphloroglucinol is presumably synthesized via monoacetyl phloroglucinol

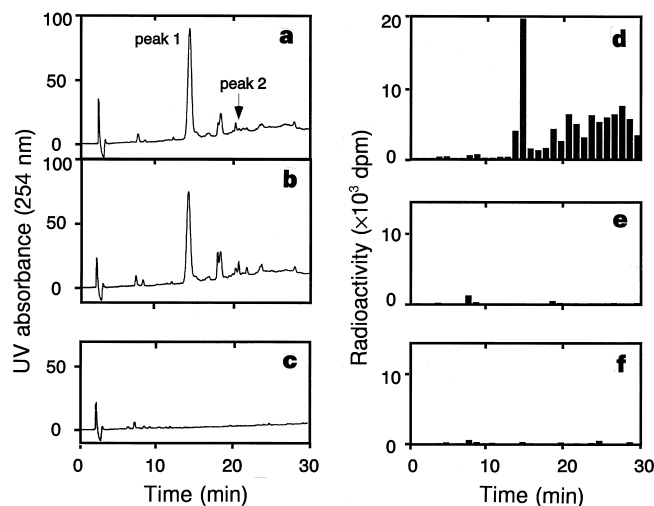


Figure 2 Reverse-phase HPLC analysis of products synthesized by RppA *in vitro*, showing absorbance at 254 nm and radioactivity of each fraction. **a, d**, Standard reaction; **b, e**, with [1-¹⁴C]acetyl-CoA; **c, f**, with the RppA/C138S mutant. Peaks 1 and 2 represent THN and flaviolin, respectively. Other minor peaks observed probably represent oxidized products derived from THN and flaviolin.

from acetyl-CoA and three molecules of malonyl-CoA with a CHS-type ring-folding of the tetraketide intermediate by the action of the RppA homologue with GCHS2-like activity, as proposed². In vancomycin biosynthesis, the 3,5-dihydroxyphenylglycine moiety is supposedly formed by the CHS activity of the RppA homologue¹⁴. We can also predict so far unknown biosynthetic pathways for naphthoquinone rings in secondary metabolites in *Streptomyces*; compounds such as naphterpin¹⁵, furaquinocins^{15,16}, napyradiomycin^{15,17} and marinone¹⁸ are biosynthesized via THN formed by RppA-related enzymes from five molecules of malonyl-CoA. Figure 3b shows examples of napyradiomycin and debromomarinone. Together with the finding that RppA is involved in melanin production (see below), it is thus apparent that the RppA enzymes (that is, CHS-related polyketide synthases) are involved in the biosynthesis of a wide variety of secondary metabolites in not only filamentous *Streptomyces* but also single-cell bacteria.

To determine the *in vivo* function of *rppA*, we disrupted the chromosomal *rppA* gene in *S. griseus* by gene replacement (see Methods). The *rppA*-disruptants grew normally and formed spores, indicating that *rppA* has no effects on growth or morphological development. However, the substrate mycelium of the disruptants contained no melanin-like pigments, whereas that of the wild-type strain contained light brown pigment (Fig. 4). Spores of the wild-type strain, which were collected through cotton, are slightly greenish, whereas those of the disruptants are creamy. Thus, disruption of *rppA* generates an 'albino'-type mutant. Introduction of pIJ486-RB44 containing *rppA* into the disruptants by transformation restored pigmentation, although it caused overproduction of the pigments in hyphae and spores. Distribution of *rppA* homologues in a wide variety of streptomycetes³ indicates that RppA homologues are key enzymes in one of the general melanin biosynthetic pathways in this bacterial genus. Note, however, that thick spore colours in some streptomycetes, such as *S. coelicolor* A3(2), *S. lividans* and *S. halstedii*, are due to polyketide pigments resulting from type II polyketide synthases^{19,20}. In some fungi, on the other hand, THN as an intermediate in the melanin biosynthetic pathway^{21–23} is biosynthesized by type I polyketide synthases. □

Methods

Production and purification of RppA in *E. coli*. General recombinant DNA techniques were as described for *E. coli*²⁴ and *S. griseus*²⁵. A 1.5-kb *Hind*III–*Bam*HI fragment containing the whole *rppA* sequence was excised from pIJ486-RB44 and cloned in pUC19 (plasmid pUC19-RB44). The nucleo-

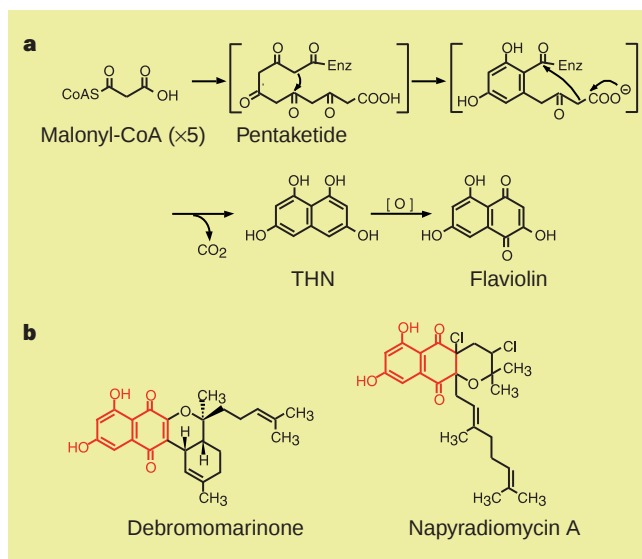


Figure 3 Biosynthesis of THN by RppA. **a**, RppA-catalysed synthesis of THN; **b**, naphthoquinone moieties (shown in red) in debromomarinone and napyradiomycin, probably synthesized by RppA-like enzymes.

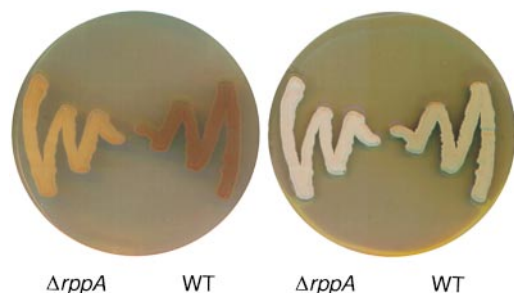


Figure 4 Phenotypes of the *rppA*-disrupted strain of *S. griseus*. Photographs were taken after growth for 4 days at 28°C. The lower (left) and upper (right) surfaces are shown. WT, wild-type.

tide sequence (CCCATG) covering the ATG start codon of *rppA* was changed to CATATG to create an *NdeI* site by PCR with primer I: 5'-GCCAAGCTT CATATGGCGACCCTGTGCCGACC-3' (italic indicates bases to be changed; underline indicates an *HindIII* site) and primer II: 5'-GGAGCCGTGCGCT CCAGGC-3' (the nucleotide sequence corresponds to the region encoding Arg 206 to Ser 212). After amplification by PCR under the standard conditions, the *HindIII*-*SphI* fragment (an *SphI* site is present within the amplified fragment) was prepared and cloned between the *HindIII* and *SphI* sites of pUC19-RB44, resulting in pUC19-RB44-N. The absence of undesired alterations was checked by nucleotide sequencing. The *rppA* sequence was then excised as an *NdeI*-*BamHI* fragment and inserted between the *NdeI* and *BamHI* sites of pET-16b (Novagen), resulting in pET-RppA. The expression plasmid pET-RppA would direct the synthesis of protein (calculated $M_r = 42.7$ K) with the structure Met-Gly-His₁₀-Ser₂-Gly-His-Ile-Glu-Gly-Arg-His-RppA. *E. coli* BL21 (DE3), harbouring pET-RppA, was grown at 26°C overnight in Luria broth containing 100 µg ml⁻¹ ampicillin, and a cell lysate was prepared by sonication. Cell debris was removed by centrifugation at 10,000 g for 20 min and the cleared lysate was applied to a column with His-bind resin (Novagen). Histidine-tagged RppA was eluted with a linear gradient of 60–600 mM imidazole. Protein concentrations were measured with a BioRad protein assay kit using bovine serum albumin as the standard. A Superose gel filtration column (12 HR 10/30; Pharmacia) equipped in an FPLC system was used to determine molecular mass.

Site-directed mutagenesis. Cys 138 of RppA was replaced with Ser using the Takara *in vitro* mutagenesis kit (Mutan-Super Express Km). The oligonucleotide used for site-directed mutagenesis was 5'-CCCATGCCCCAATTGGGC-TCTGCGGCGG-3' (italic letters represent a Ser codon). Part of the *rppA* sequence containing the region to be mutagenized was excised from pUC19-RB44-N and used as the template. The mutation and the absence of undesired alterations were confirmed by nucleotide sequencing. The mutant RppA protein was similarly purified with the His-bind resin.

RppA assay and identification of the products. The standard reaction contained, in a final volume of 1 ml; 100–120 µg RppA, 0.2 mM [2-¹⁴C]malonyl-CoA (2.2 mCi mmol⁻¹, 9.8 × 10⁵ dpm; Dupont), 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM 2-mercaptoethanol. When 0.2 mM [1-¹⁴C]acetyl-CoA (2.2 mCi mmol⁻¹, 9.8 × 10⁵ dpm; Dupont) was used instead of [¹⁴C]malonyl-CoA, we added 0.2 mM malonyl-CoA. Conversion of *p*-coumaroyl-CoA was tested by adding it at a final concentration of 0.15 mM to the standard reaction. Reactions were carried out for 1 h at 30°C and stopped by adding 200 µl of 6 N HCl. The mixture was extracted with ethyl acetate and the solvent was evaporated. The resultant materials were dissolved in methanol and analysed by reverse-phase HPLC to determine the distribution of radioactivity. Reverse-phase HPLC conditions: ODS-80Ts column (4.6 × 150 mm; Tosoh), maintained at 40°C; mobile phase, linear from 5% CH₃CN in H₂O (each contained 2% acetate) to 40% CH₃CN in H₂O over 30 min with detection at 254 nm; flow rate, 0.8 ml min⁻¹. We measured the radioactivity of each fraction directly by liquid scintillation counting.

LC-APCIMS. LC-APCIMS was measured on LCQ (Thermo Quest) under the same conditions as for HPLC equipped with a reverse-phase ODS-80Ts column. The authentic sample of THN and the compound in peak 1 gave the same LC profile and MS pattern on LC-APCIMS. Retention time was 14 min on LC. Multi-stage LC-APCIMS (positive): THN MS 193 [M + H]⁺; MS/MS (precursor ion at

m/z 193), 175, 165, 151, 147, 137, 123, 119. Multi-stage LC-APCIMS (negative): MS 191 [M - H]⁻; MS/MS (precursor ion at m/z 191), 147, 123.

Gene disruption. Between the *Tth111I* site (corresponding to Val 38) and the *HincII* site (corresponding to Val 254) within the *rppA*-coding sequence, a 1.32-kb *SmaI* fragment containing the kanamycin-resistance (*aphII*) determinant on Tn5 (ref. 26) was inserted. A DNA fragment containing the mutated *rppA* sequence, together with 3.3-kb upstream and 2.2-kb downstream regions, was cloned in pUC19. The pUC19 plasmid was digested with *DraI*, denatured with NaOH²⁷ and introduced by protoplast transformation into the wild-type strain *S. griseus* IFO13350. Candidates for *rppA*-disruptants were selected as kanamycin-resistant colonies. Eight disruptants with correct gene replacement as a result of double cross-over were chosen by Southern hybridization with the chromosomal DNA as the target and the *aphII* sequence and the intact *rppA* sequence as the ³²P-labelled probes. We examined pigmentation of the disruptants on YMPD agar medium²⁸.

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- Schröder, J. A family of plant-specific polyketide synthases: facts and predictions. *Trends Plant Sci.* **2**, 373–378 (1997).
- Schröder, J. *Comprehensive Natural Products Chemistry* Vol. 1 (ed. Sankawa, U.) 749–771 (Elsevier, Amsterdam, 1999).
- Ueda, K., Kim, K.-M., Beppu, T. & Horinouchi, S. Overexpression of a gene cluster encoding a chalcone synthase-like protein confers reddish brown pigment production in *Streptomyces griseus*. *J. Antibiot.* **48**, 638–646 (1995).
- Tropf, S., Kärcher, B., Schröder, G. & Schröder, J. Reaction mechanisms of homodimeric plant polyketide synthases (stilbene and chalcone synthase). *J. Biol. Chem.* **270**, 7922–7928 (1995).
- Lanz, T. et al. The role of cysteine in polyketide synthases. Site-directed mutagenesis of resveratrol and chalcone synthases, two key enzymes in different plant-specific pathways. *J. Biol. Chem.* **266**, 9971–9976 (1991).
- Fujii, I. et al. Heterologous expression and product identification of *Colletotrichum lagenarium* polyketide synthase encoded by the PKS1 gene involved in melanin biosynthesis. *Biosci. Biotechnol. Biochem.* **63**, 1445–1452 (1999).
- Eckermann, S. et al. New pathway to polyketides in plants. *Nature* **396**, 387–390 (1998).
- Dimroth, P., Ringelmann, E. & Lynen, F. 6-Methylsalicylic acid synthase from *Penicillium patulum*. Some catalytic properties of the enzyme and its relation to fatty acid synthase. *Eur. J. Biochem.* **68**, 591–596 (1976).
- Hopwood, D. A. Genetic contributions to understanding polyketide synthases. *Chem. Rev.* **97**, 2465–2497 (1997).
- Hutchinson, C. R. & Fujii, I. Polyketide synthase gene manipulation: a structure–function approach in engineering novel antibiotics. *Annu. Rev. Microbiol.* **49**, 201–238 (1995).
- Katz, L. & Donadio, S. Polyketide synthesis: prospects for hybrid antibiotics. *Annu. Rev. Microbiol.* **47**, 875–912 (1993).
- Bangera, M. G. & Thomashow, L. S. Characterization of a genomic locus required for synthesis of the antibiotic 2,4-diacetylphloroglucinol by the biological control agent *Pseudomonas fluorescens* Q2-87. *Mol. Plant–Microbe Interact.* **9**, 83–90 (1996).
- van Wageningen, A. M. A. et al. Sequencing and analysis of genes involved in the biosynthesis of a vancomycin group antibiotic. *Chem. Biol.* **5**, 155–162 (1998).
- Hammond, S. J. et al. On the biosynthesis of the antibiotic vancomycin. *J. Chem. Soc. Chem. Commun.* **1982**, 344–346 (1982).
- Shin-ya, K., Furihata, K., Hayakawa, Y. & Seto, H. Biosynthetic studies of naphterpin, a terpenoid metabolite of *Streptomyces*. *Tetrahedron Lett.* **31**, 6025–6026 (1990).
- Funayama, S., Ishibashi, M., Komiya, K. & Omura, S. Biosynthesis of furaquinocins A and B. *J. Org. Chem.* **55**, 1132–1133 (1990).
- Shioiri, K. et al. Structures of new antibiotics napyradiomycins. *J. Antibiot.* **39**, 494–501 (1986).
- Pathirana, C., Jensen, P. R. & Fenical, W. Marinone and debromomarinone: antibiotic sesquiterpenoid naphthoquinone of a new structure class from a marine bacterium. *Tetrahedron Lett.* **33**, 7663–7666 (1992).
- Blanco, G. et al. Hybridization and DNA sequence analyses suggest an early evolutionary divergence of related biosynthetic gene sets encoding polyketide antibiotics and spore pigments in *Streptomyces* spp. *Gene* **130**, 107–116 (1993).
- Yu, T.-W. et al. Engineered biosynthesis of novel polyketides from *Streptomyces* spore pigment polyketide synthases. *J. Am. Chem. Soc.* **120**, 7749–7759 (1998).
- Bell, A. A. & Wheeler, M. H. Biosynthesis and functions of fungal melanins. *Annu. Rev. Phytopathol.* **24**, 411–451 (1986).
- Mayorga, M. E. & Timberlake, W. E. The developmentally regulated *Aspergillus nidulans* *wA* gene encodes a polyketide homologous to polypeptide and fatty acid synthases. *Mol. Gen. Genet.* **235**, 205–212 (1992).
- Takano, Y. et al. Structural analysis of *PKS1*, a polyketide synthase gene involved in melanin biosynthesis in *Colletotrichum lagenarium*. *Mol. Gen. Genet.* **249**, 162–167 (1995).
- Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1982).
- Hopwood, D. A. et al. *Genetic Manipulation in Streptomyces: A Laboratory Manual* (The John Innes Foundation, Norwich, UK, 1985).
- Beck, E. et al. Nucleotide sequence and exact localisation of the neomycin phosphotransferase gene from transposon Tn5. *Gene* **19**, 327–336 (1982).
- Oh, S. H. & Chater, K. F. Denaturation of circular or linear DNA facilitates targeted integrative transformation of *Streptomyces coelicolor* A3(2): possible relevance to other organisms. *J. Bacteriol.* **179**, 122–127 (1997).
- Ando, N., Ueda, K. & Horinouchi, S. A *Streptomyces griseus* gene (*sgaA*) suppresses the growth disturbance caused by high osmolality and a high concentration of A-factor during early growth. *Microbiology* **143**, 2715–2723 (1997).

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This free poster from Pierce displays the reactive chemistries and linkages created by the company's EZ-Link biotinylation reagents. Reagents featured include a new one, EZ-Link TFP-PEOBiotin, an amine-reactive reagent with a long (32.6 Å) hydrophilic spacer arm. The tetrafluorophenyl ester (TFP) reacts with primary and secondary amines in aqueous buffers from pH 7 to 9. The polyethylene oxide spacer (PEO) arm makes this compound more water-soluble than PFP-Biotin. For a free poster call 800-874-3723 (US). Elsewhere, call the local distributor or use e-commerce.

Reader Service No. 106

Proteasome inhibitor

From Affiniti Research www.affiniti-res.com
A peptidyl boronic acid inhibitor for proteasome system research

Specific inhibitors of proteasome activity are vital to work on its function. Z-Leu-Leu-Leu-H (also known as MG-132) is a leupeptin analogue which inhibits proteasomal chymotrypsin-like activity. It also induces apoptosis in MOLT-4 and L5178Y cells via a p53-dependent pathway and enhances CPP32-like activity in TNF-treated U937 cells.

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Isoquant

From Promega www.promega.com
Rapid measurement protein deamidation

Now available from Promega is a nonradioactive protocol for the Isoquant Protein



Detect it: Isoquant keeps track of deamidation.

Deamidation Detection Kit. With fewer steps than the radioactive assay, this protocol using reverse-phase HPLC, reduces test time to less than an hour and eliminates costs and hazards associated with radioactive materials. The kit provides a tool to track the formation of isoaspartic acid residues in proteins by measuring the amount of methyl donor used in an enzymatic methylation reaction.

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L-8800 analyser

From Hitachi www.hsi-europe.com
High-speed amino acid analysis

Supplied complete with a PC for control and data analysis, the L-8800 amino acid analyser offers high speed operation at picomole detection levels. The L-8800 is built around a newly designed reaction column, featuring a special 3- μ m ion-exchange resin. This column can be repacked and regenerated completely. The new column offers significant time-savings over conventional HPLC methods. Analysis of protein hydrolysate can take as little as 20 min compared to 50 min by HPLC and analysis of physiological fluid can take as little as 70 min compared to 135 min by HPLC.

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Tyrosine kinase assay

From PanVera Corporation www.panvera.com
Available in 100- and 1,000-assay packs

This novel *in vitro* assay was developed for the detection of tyrosine kinase activity and

rapid screening of novel protein tyrosine kinase inhibitors. The Pan Vera Protein Tyrosine Kinase Assay is a non-radioactive, homogeneous assay for use with single tubes using the Beacon 2000 FP instrument or with 96- or 384-well plates using commercially available HTS instrumentation.

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Xplore mRNA

From Endogen Inc. www.endogen.com

A rapid yet sensitive mRNA assay.

Endogen's Xplore mRNA Quantification Systems require 500 ng or less of total RNA and permit detection of as little as 1 attomole of the molecule of interest. This is equivalent to 1–5 copies of mRNA per cell in 500,000 cells. The assay utilizes a pair of probes that bind to a splice junction on the specific mRNA targeted. The probe/RNA complex is recognized by a novel repair endonuclease, Cleavase VII enzyme, which catalyses a rapid, isothermal, signal amplification reaction. The reaction product is captured on a streptavidin-coated plate via a biotin-labelled oligonucleotide. Assays for human IL-1 β , IL-2, IL-4, IL-6, IL-10, IFN- γ and TNF- α , together with separate β -actin and GAPDH control assays are available.

Reader Service No. 111

Affinity 98

From Molecular Simulations www.msi.com

Automatic docking of ligands to protein receptors

The latest release of Affinity, MSI's automated flexible docking software, allows the receptor binding site to relax during docking, then automatically finds the best binding modes of the ligand to the receptor. This improved method incorporates a Simulated Annealing protocol for refinement of the bound ligand and the ability to include or remove water molecules and specific hydrogen bonding patterns during docking. Affinity is especially useful in structure-based drug design where the experimentally determined structure of a protein-ligand complex is often unavailable.

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Miniaturize it: Bio-Rad shrinks electrophoresis.

Mini-Protein 3

From Bio-Rad www.bio-rad.com

Simplify the handling and running of precast and handcast gels

Bio-Rad's new mini vertical electrophoresis system can be put together in only 30 seconds. Evolved from the Mini-Protein II, the Mini-Protein 3 eliminates the time-consuming assembly procedures often associated with electrophoresis applications. The new hand casting module features a simple lock mechanism. The new combs are made of plastic, are easier to handle and are labelled with thickness and number of wells. To ensure more reproducible well formation, ridges on the combs completely seal off the acrylamide in the gel sandwich to eliminate air and aid polymerization.

Reader Service No. 113

Inhibitory cocktails

From Sigma www.sigma.sial.com

Four new inhibitor cocktails for protein isolation studies

These phosphatase cocktails from Sigma contain inhibitors of tyrosine protein phosphatases, acid and alkaline phosphatases, serine/threonine phosphatases and L-isoenzymes of alkaline phosphatase. The inhibitory action of these cocktails has been tested against phosphatase activities found in human placenta, bovine liver, rabbit muscle, A431 or Jurkat cell extracts. Of the two special protease inhibitor cocktails, one is

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formulated for studies of plant cell extracts and the other for reaction with poly-histidine tagged proteins found in cell extracts of *E. coli* and baculovirus infected cells.

Reader Service No. 114

IonClear BigBead

From Sterogene www.sterogene.com

Get ready for ion-exchange chromatography

IonClear BigBead reduces the ionic strength of protein solutions in preparation for ion-exchange chromatography. It allows for the direct capture of antibodies and proteins from fermentation and cell culture harvest without the need for a 3–5 fold dilution. The idea is to replace diafiltration with a more gentle ion adsorption process. Recoveries and biological activities are typically above 99%. IonClear typically works in 5–10 minutes, reducing processing times

relative to diafiltration by a factor of 10–100 fold.

Reader Service No. 115

Fluo-peptides

From NEN www.nenlifesci.com

Fluorescent peptides in receptor binding assays

A new brochure from NEN Life Science Products describes Fluo-peptides, biologically active fluorescent peptides for use in characterization of G protein-coupled receptors (GPCRs). The brochure describes their use in cellular imaging, flow cytometry/ FACS, and fluorescence polarization receptor binding assay applications. Developed and manufactured by Advanced Bioconcept, Fluo-peptides are a line of peptides conjugated with fluorophores in a way that maintains their biological activity.

Reader Service No. 116

Dynabeads

From Dynal

www.dynal.no

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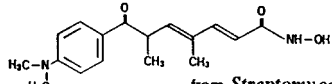
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